A NOVEL GENE-ENVIRONMENT INTERACTION: THE HUNTINGTON MUTATION SUPPRESSES MANGANESE ACCUMULATION AND TOXICITY

By

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For all those in my life that remind me of who I want to be; my parents, my Alexandria, my husband and God

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LIST OF ABBREVIATIONS

3NPA: 3-Nitroproprionic Acid

AD: Alzheimer's disease

Akt: member of the protein kinase B family of signaling molecules

ALS: Amyotrophic lateral sclerosis

APOE: apolipoprotein E

ATXN1[82Q]: Full-length Ataxin 1 construct with 82 glutamines in the polyglutamine domain

BDNF: Brian Derived Neurotrophic Factor

Cd: Cadmium

Co: Cobalt

Cu: Copper

DMEM: Dulbecco's Modified Eagle Medium

DMT1: Divalent metal transporter 1

Fe: Iron

GFAAS: graphite furnace atomic absorption spectroscopy

GS: Glutamine synthetase

HD: Huntington's disease

HEK293T: Human embryonic kidney cell line

HTT: Huntingtin gene or protein

HTT[128Q]: HTT with 128 glutamines in the polyglutamine domain

LDH: Lactate dehydrogenase

Mn: Manganese

MPP^+: 1-methyl-4-phenylpyridinium. It is the metabolite of MPTP.

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a neurotoxin.

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt

Ni: Nickel

NMDAR: N-methyl-D-aspartic acid Receptor

Pb: Lead

PD: Parkinson's disease

PrP: Prion precursor protein

RNAi: interference RNA

ROS: reactive oxygen species

SOD2: Superoxide dismutase 2

ST$Hdh^{Q111/Q111}$: Striatal cell line expressing mutant mouse huntingtin

ST$Hdh^{Q7/Q7}$: Striatal cell line expressing wild-type mouse huntingtin

TfR: Transferrin Receptor

YAC128Q: HD animal model expressing mutant huntingtin with 128 repeats

Zip8: Member of the Solute-Carrier-39 (SLC39) Metal-Transporter Family

Zn: Zinc

CHAPTER I

METALS AND HUNTINGTON'S DISEASE: THEIR POTENTIAL FOR GENE-ENVIRONMENT INTERACTIONS

Gene-Environment Interactions in Neurodegenerative Disorders

Even with conservative estimates, deaths due to neurodegenerative diseases will increase by approximately 119% as the elderly population in the United States more than doubles by 2040 (Lilienfeld et al. 1993). Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) are the most common of these progressive disorders. Each is associated with decay in movement, balance, and cognitive decline, as well as other nonmotor symptoms. HD is a monogenetic disorder in which a trinucleotide expansion in the *huntingtin* gene (*HTT*) causes disease. AD, PD, and ALS are complex disorders with various factors playing a role disease risk.

Familial forms of AD, PD, and ALS are caused by genetic mutations and are inherited in an autosomal dominant fashion similarly to HD. In AD for example, mutations in presenilin 1 and amyloid precursor protein lead to autosomal dominant inheritance of the disease with early-onset, before age 60. While, single nucleotide polymorphisms in the α-synuclein as well as mutations in parkin, UCH-L1, PINK1, DJ-1, and other genes cause inheritable forms of PD (Coppede et al. 2006). Ten percent of

ALS cases are considered familial and are caused by mutations in seven genes, such as superoxide dismutase 1 (Bossy-Wetzel et al. 2004).

Susceptibility to the more common sporadic forms of these diseases can be enhanced by environmental, epigenetic, and genetic factors (Bossy-Wetzel et al. 2004). These susceptibility factors can change risk of acquiring the disease or alter the progression of the disorder, changing the age of onset or symptom severity. Alterations in an associated gene increase the susceptibility to a disease but are not sufficient to cause the disease without other modifiers. For example, the more common late-onset form of AD is associated with alterations in apolipoprotein E (APOE). APOE represents one of over 500 genes labeled as genetic risk factors associated with this disease (Bertram et al. 2008). Polymorphisms in APOE, cytochrome P450, and monoamine oxidase B are considered susceptibility factors for acquiring sporadic PD. Though risk factors in ALS are not as clear cut as in other diseases, mutations in neurofilament and the vascular endothelial growth factor (VEGF) receptor have been associated with increased incidence of ALS (Coppede et al. 2006). The prominent genetic connections detailed above emphasize that single gene mutations do not completely explain the observed variation in patients' symptoms that can occur in these diseases (Bertram et al. 2005).

The study of environmental influences on neurological diseases is still somewhat in its infancy, but strong evidence has emerged illustrating the importance of gene-environment interactions. Environmental risks including participation in sports such as soccer or American football has been linked to ALS and PD (Coppede et al. 2006). Exposure to toxicants can also increase susceptibility to neurodegenerative disorders. One classic example is the induction of Parkinsonism by the neurotoxin 1-methyl-4-

phenylpyridinium (MPP^+), a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which kills the dopaminergic neurons of the substantia nigra (Figure I.I.A. and B.). MPTP is responsible for inducing PD in drug addicts who ingested it as a contaminate in their illicit drugs (Johannessen et al. 1984). Additionally, some pesticides, such as paraquat, also contain a toxin similar to MPP^+, which has been linked to PD (Cicchetti et al. 2009). Both the pesticide and MPP^+, inhibit mitochondrial complex I function and lead to cell death (Tansey et al. 2007).

Diseases that are clearly genetic such as HD are even susceptible to modification by environmental factors. Studies of monozygotic twins have shown significant variability in HD progression between siblings (Georgiou et al. 1999). Additionally, studies in HD and other neurodegenerative diseases have reported exercise and enrichment is beneficial slowing the disease progression (Carreras et al. ; Kramer et al. 1999; van Dellen et al. 2008; Nichol et al. 2009)

The Neurotoxicity of Metals

Metals are environmental agents associated with neurodegeneration, and exposures to high levels of metal ions have well-defined toxicities within the brain (Bossy-Wetzel et al. 2004; Brown et al. 2005; Coppede et al. 2006). Additionally, multiple neurodegenerative disorders have been associated with altered metal homeostasis emphasizing the importance of metals for normal brain function.

Altered levels of copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), and other trace metals have been reported as elevated in neurodegenerative diseases that are not the

result of overexposure to these metals. For example, Cu and Fe are all reported elevated in patients' brains with HD (Dexter et al. 1991; Dexter et al. 1992; Perl et al. 2007). The brains of PD patients also show increases in Fe (Dexter et al. 1991). Diseases of metals such as Wilson's disease and neuroferritinopathy can also lead to neurodegeneration. These two diseases are both caused by genetic mutations that lead to improper storage of Cu or Fe respectively (Cozzi et al. 2006; Madsen et al. 2007).

Various metals (e.g. Mn, Cu, Zn, and aluminum (Al)), have been found to be associated with protein aggregates of multiple neurodegenerative diseases. Exposure to metals can increase the rate of amyloidogenesis or aggregate formation (Jobling et al. 2001; Uversky et al. 2001; Binolfi et al. 2006). Cu, for example, binds prion precursor protein (PrP), altering the conformation of the protein (Gaeta et al. 2005). Additionally, Mn has been shown to promote aggregation without binding to PrP (Levin et al. 2005). Proteins with the propensity to form inclusions are thought to accumulate through a similar process called amyloidogenesis. One theory is that the process is facilitated by metals due to macromolecular crowding. The theory of macromolecular crowding posits that limiting space in the cytosol of the cell with any item (e.g. proteins or metals) can alter the viscosity and the cytosolic volume available for molecules to move leading to aggregation of some proteins (Munishkina et al. 2004). Given that Mn can induce a parkinsonian-like disease and induce aggregation of α-synuclein, it is important to note that Lewy bodies, the aggregates commonly formed in PD, may not form in cases of Manganism-related PD (Perl et al. 2007).

Finally, metals may modify the severity of neurological diseases by increasing oxidative stress (Gaeta et al. 2005). Oxidative stress, a by-product of normal oxygen

consumption, causes cellular damage as reactive oxygen species (ROS) alter DNA, RNA, and proteins. Fe, Cu, and Mn have all been shown to increase ROS within the cell (Chen et al. 2006; Molina-Holgado et al. 2007; Flora et al. 2008). With oxidative stress already being implicated as a primary toxic mechanism in most neurodegenerative diseases, exposure to any reagent that increases this stress could push the defective system towards death (Beal 1998; Castilho et al. 1999; Bartzokis et al. 2000; Alcaraz-Zubeldia et al. 2001; Hazell et al. 2006).

Not only do metals have the potential to alter neurodegenerative diseases, they are also toxic. Metals exist naturally in the environment and can easily become concentrated in water supplies, allowing for bioaccumulation. The neurotoxicity directly associated with metal exposure can be seen in Minamata Bay, Japan. For over thirty years, water supplies were contaminated by methyl mercury dumped into the bay by local industry. The metal bioaccumulated in the fish and led to poisoning of thousands of people whose diets predominantly include fish (Harada 1995). Mn overexposure can also lead to a neurological condition similar to idiopathic PD called manganism (Compare Figure I.1. and I.2.A.) (Perl et al. 2007). Current studies in the United States have found increased levels of Mn in families living near a ferromanganese refinery (Haynes et al. 2009). The effect of the exposure in this population is currently being studied, but Mn toxicity has long been considered an industrial hazard. The brain concentrates specific metals, including Mn, to various regions leading to toxicity in these areas. Mn, for example, accumulates in the basal ganglia, specifically the globus pallidus, substantia nigra pars reticulata, the caudate nucleus and putamen of the striatum. The dopamine neurons or

their projections are lost in these brain regions likely causing the movement disturbances seen in manganism (Yamada et al. 1986; Olanow et al. 1996).

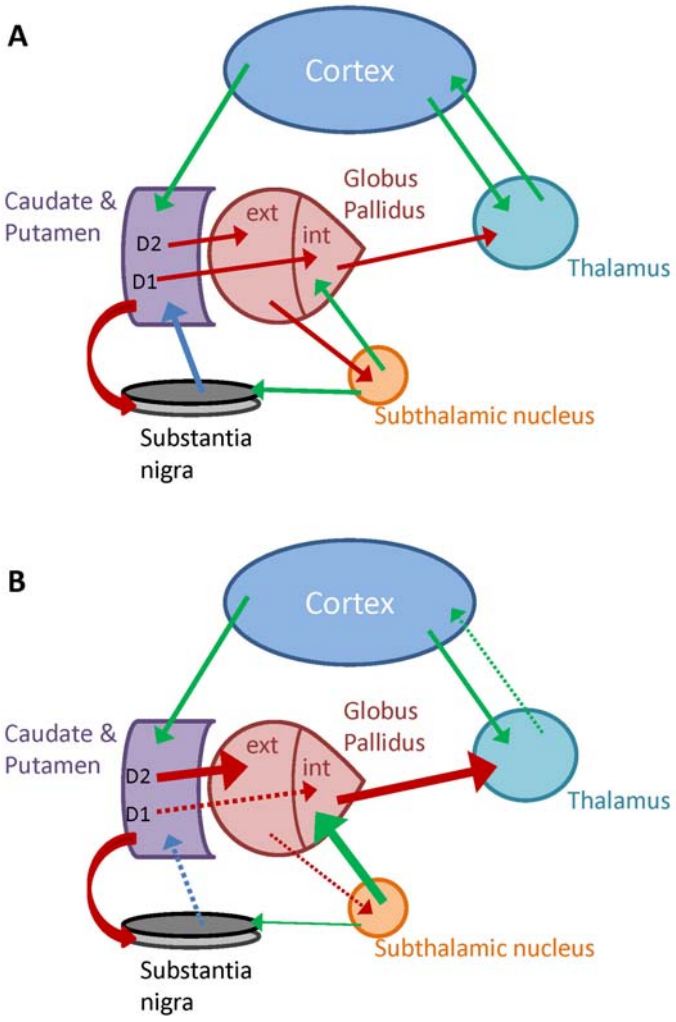


Figure I.1. Schematic of Basal Ganglia Circuitry: A. Normal B. Altered in Parkinson's Disease. (Note the decreased output from the substantia nigra to the caudate and putamen.) Blue arrows represent dopamine signaling, Red arrows represent GABA signaling, and Green arrows represent Glutamate signaling. Modified from http://thalamus.wustl.edu/course/cbell6.gif.

Manganese

Overview

Mn is an essential trace element being required for proper growth and survival. Mn is found ubiquitously throughout the body and plays a role in the function of multiple organs. Taken into the body primarily through food, its absorption is tightly controlled; both the digestive track and the blood brain barrier regulate the accumulation of this metal. For example, only 3-5% of the Mn ingested moves into the blood and the remainder is excreted (Au et al. 2008). Once in the blood, Mn must cross the restrictive blood brain barrier to enter the brain. Extreme forms of environmental exposure do exist leading to a neurodegenerative condition, Manganism (Figure I.2.A.). However, exposure levels considered normal may influence the progression of other neurodegenerative diseases.

Mn-Dependent Metalloenzymes

Mn is an essential cofactor for various enzyme families, regulating amino acid, lipid, and both protein and carbohydrate metabolism. In the brain, important examples of these essential metalloenzymes are arginase, glutamine synthetase, pyruvate decarboxylase, superoxide dismutase 2 (SOD2), and serine/threonine protein phosphatase. Deficiencies in Mn are rare; in fact, other metals such as Cu and magnesium can substitute for Mn keeping many enzymes functional (Christianson 1997;

Takeda 2003). Mn deficiency can lead to a wide range of problems including birth defects (e.g. skeletal malformations and impaired growth), seizures, and abnormal glucose tolerance (Keen et al. 1999).

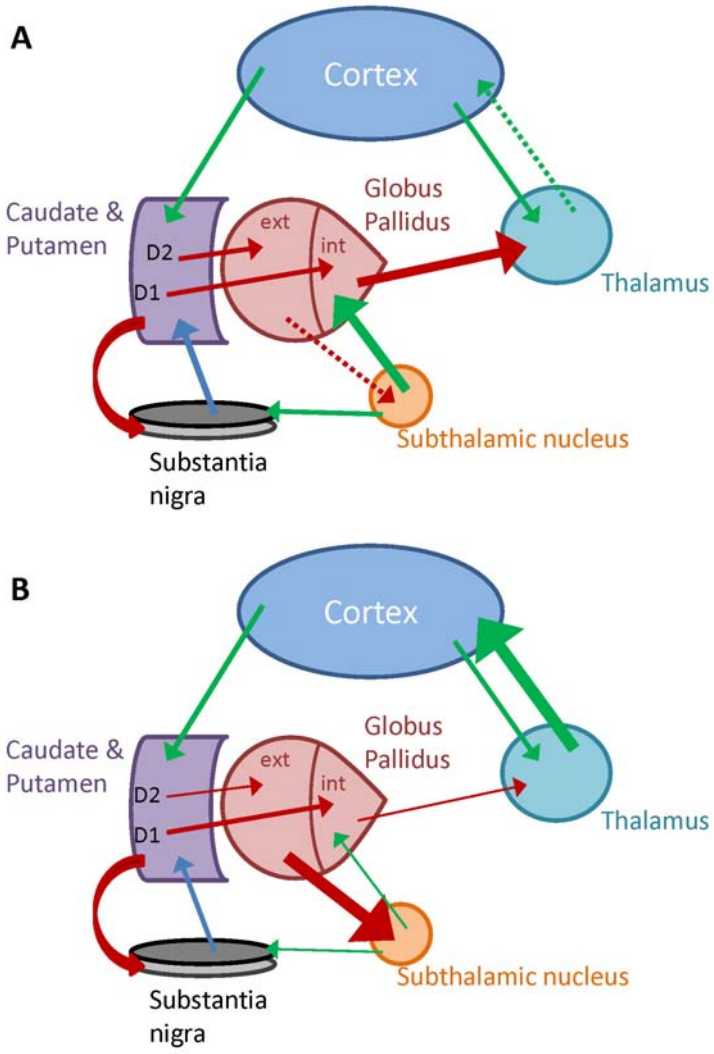


Figure I. 2. Schematic of Basal Ganglia Circuitry: A. Altered in Manganism (Note loss of GABA signal from Globus Pallidus leading to less signal from the thalamus) **B. Altered in Huntington's Disease**.(note loss of D2 signal leads to increased signal from Thalamus). Blue arrows represent dopamine signaling, Red arrows represent GABA signaling, and Green arrows represent Glutamate signaling. Modified from http://thalamus.wustl.edu/course/cbell6.gif.

Indeed, brain disorders have been linked to Mn dependent enzymes. Epileptic seizures can be associated with a reduction in glutamine synthetase activity (Eid et al. 2008). Loss of SOD2 activity is known to cause increases in oxidative stress and increase the toxicity of mitochondrial respiratory complex inhibitors (Andreassen et al. 2001). Proper neurotransmitter signaling is regulated by various Mn-dependent enzymes. Nitric oxide signaling is regulated by arginase, and glutamine synthetase regulates the cycling of glutamate to glutamine (Estevez et al. 2006; Maciejewski et al. 2008).

Mn Transport

How Mn is transported is not thoroughly understood, and no transport system has been identified that moves only Mn. The shared transporters can also move other metals such as Fe, Co, Cu, Zn, nickel (Ni), cadmium (Cd), and lead (Pb). Most Mn is thought to cross the blood brain barrier via several mechanisms including facilitated diffusion and active transport mechanisms. The divalent metal transporter (DMT1) along with calcium (Ca) and Fe transporters are known to shuttle Mn (Murphy et al. 1991; Rabin et al. 1993; Aschner et al. 1994; Gunshin et al. 1997; Garrick et al. 2003).

Fe transport is tightly linked to the movement of Mn. The transferrin receptor (TfR) moves Mn in the 3+ valence, but has higher affinity for Fe^{3+} (Malecki et al. 1999). Not only can trivalent Mn be transported by the TfR but brain Fe levels can alter Mn transport (Keefer et al. 1970; Aschner et al. 1994; Erikson et al. 2004; Fitsanakis et al. 2008). DMT1, which transports Mn^{2+} can be regulated by Fe concentrations. There are two isoforms of DMT1 created by alternative splicing. One isoform is thought to be

regulated by Fe, likely via the IRE sequence of 25 nucleotides within the 3' untranslated region of the gene (Fleming et al. 1998; Lee et al. 1998). The expression of this transporter is ubiquitous but not uniform. Within the brain, the most densely stained areas are in the caudate nucleus, putamen, and substantia nigra pars reticulata, the regions most susceptible to Mn toxicity (Huang et al. 2004). Electrophysiological studies indicate that DMT1 acts as a symporter moving both protons and metals across the membrane (Gunshin et al. 1997; Garrick et al. 2003). Additionally, the transporter can colocalize with the TfR and removes the bound metal from the acidic endosome after it dissociates from the TfR. (Gruenheid et al. 1999).

The citrate transporter and a metal/bicarbonate ion symporter, ZIP-8, have been shown to transport Mn (Davidsson et al. 1989; Aschner et al. 1994; Crossgrove et al. 2003; He et al. 2006). The mechanism or relevance of the citrate transporter in relation to Mn shuttling is not fully understood. Zip-8 functions by transporting HCO_3^- along with Mn (He et al. 2006). Other Mn transporters are voltage-gated, store-operated, and ionotropic glutamate receptor calcium channels (Lucaciu et al. 1997; Kannurpatti et al. 2000; Riccio et al. 2002).

Huntington's Disease

Overview

HD is a progressive neurological disorder that affects approximately 1 in 10,000 individuals worldwide. One of the first descriptions of HD was in 1872 by George Huntington (Huntington 1872). The disorder is characterized by both cognitive and movement abnormalities involving both voluntary and involuntary muscle groups. The most common emotional symptom of the disease is depression; however, irritability, apathy, and anxiety can also accompany the choreiform movements of the head, neck, and extremities. HD is fatal, leading to death 10-20 years after the first symptoms appear. Tragically, HD symptoms usually do not appear in an affected individual until their mid-thirties, after prime childbearing years have already passed and the disease causing mutation has been inherited by the next generation. HD is an autosomal dominant disorder, with each child having a 50/50 chance of inheriting the mutation from his/her affected parent. Many patients who have already witnessed and cared for a parent dying from HD choose suicide to spare their loved ones from participating in their decline. The disease is known to be caused by an expansion in a normal trinucleotide (CAG) repeat in the *HTT* gene (Cattaneo et al. 2005; Imarisio et al. 2008; Damiano et al. 2009).

Unfortunately, medical science has limited resources to offer HD patients. Clinical treatments focus on controlling the symptoms caused by the disease but to date no treatment to stop disease progression exists. Drugs which block dopamine receptors

were traditionally used to treat the motor symptoms; however, more recently tetrabenazine, which blocks the uptake of dopamine in to vesicles leading to its degradation, has been approved to control the jerky motor movements (Adam et al. 2008). However, these medications are not recommended for those suffering from with depression. Tranquilizers and antipsychotic drugs have also been used to control the symptoms but also are riddled with side effects (Jankovic 2009).

HD Pathology

With no current ability to halt the disease progression, it is surprising that we understand so many of the details of the disease. The genetic mutation detailed below leads to a progressive degeneration of multiple brain regions, focusing initially on the D2 receptor-positive, medium spiny neurons (MSN) of the striatum. The loss of these neurons produces the defining motor abnormalities associated with HD. The striatum is a key player in the motor circuitry of the brain controlling excitation of the cortex. In HD, the loss of the inhibitory or GABAergic MSN allows overstimulation of the cortex and leads to the production of the uncontrollable movements (Figure I.2.B). As the disease progresses, other brain regions such as the cortex, globus pallidus, subthalamic nuclei, substantia nigra, cerebellum, and the thalamus, also degenerate. The neurodegeneration in the cortex is predominately a loss of pyramidal neurons (Cowan et al. 2006). The molecular causes of this selective cell loss are still unknown.

Wild-type HTT Function

The function of the normal HTT protein is poorly understood, thus limiting our ability to understand the progression of HD. HTT associates with over 200 proteins and has a key role in regulating transcription factors such as RE1/NRSE (Zuccato et al. 2003). Due to its large size (~350kDa) and ubiquitous expression pattern, it has been challenging to pinpoint a function. Additionally, the protein has no conserved sequence homology with other proteins that can help us elucidate its function (Harjes et al. 2003). Several structural motifs have been identified, but their roles in HTT function remain unclear. The polyglutamines at the amino-terminus of the protein are expanded in the disease state with more than 35 glutamines leading to the disorder. The polyglutamine expansion is then followed by the polyproline rich domain that is thought to play a role in making HTT soluble. The other major structural element of HTT is a series of HEAT (Huntingtin, Elongation factor 3, protein phosphatase 2A, TOR1) repeats. The 28-36 HEAT repeats are thought to play a role in protein scaffolding interactions (Andrade et al. 1995; Takano et al. 2002). The wild-type protein is expressed throughout the cell, associated with membranes, mitochondria, and microtubules. It is also reported to be in a soluble form within the cytosol and the nucleus (Hoogeveen et al. 1993; Trottier et al. 1995; Persichetti et al. 1996; Ferrante et al. 1997; Velier et al. 1998). A functional nuclear export sequence is at the carboxy-terminus of the protein, and it contains a potential nuclear localization site. Studies have also focused on the post-translational modifications of the protein such as phosphorylation, palmitoylation, and protein cleavage by both caspases and calpains linking these modifications to protein aggregation, vesicles transport, and cell death (Figure I.3.) (Kalchman et al. 1996;

Humbert et al. 2002; Steffan et al. 2004; Cattaneo et al. 2005; Warby et al. 2005; Imarisio et al. 2008; Warby et al. 2009).

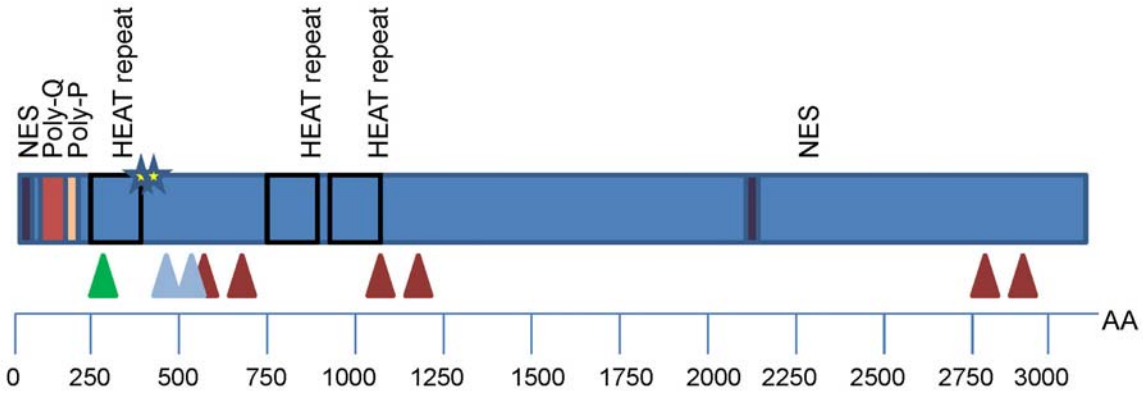


Figure I.3. Schematic of HTT Protein Amino Acid Sequence. Marking indicate the following: NES (nuclear export sequence), Poly-Q (polyglutamine domain), Poly-P (polyproline rich area), Black boxes (HEAT repeat clusters), Star (phosphorylation sites), Green arrow (palmitoylation site), Blue arrows (calpain cleavage sites), Red arrows (caspase cleavage sites). Modified from (Cattaneo et al. 2005).

Fortunately, orthologs of the human protein exist in all animal systems. Genetic studies in animals have shown that *HTT* mouse knockouts are embryonic lethal early in development (in mouse E7.5) at the stage of gastrulation (Duyao et al. 1995; Zeitlin et al. 1995). Early death also occurs in the zebra fish knockdown of HTT, due to a defect in cellular Fe acquisition (Lumsden et al. 2007). Hypomorphic alleles in the mouse, with a 50% reduction in HTT expression display altered brain development and neurogenesis resulting in prenatal death (White et al. 1997). Adult mice with *HTT* inactivated in post-mitotic cells display increased cell death (Dragatsis et al. 2000). RNAi knockdown of HTT in mouse and *Drosophila melanogaster* have shown severe alterations in organelles and vesicle transport (Gauthier et al. 2004; Trushina et al. 2004). Overexpression of the

wild-type protein has shown clearly that it limits apoptosis under a number of conditions including when expressed alongside mutant HTT (Hackam et al. 2000; Rigamonti et al. 2000). The function of wild-type HTT is crucial for normal development and neuronal health, but it is not clear how the expansion of the polyglutamine domain alters one or more of the functions of the protein. The role of HTT in development may indicate a dual function of the protein as mutant HTT can recover the embryonic lethality (White et al. 1997).

The Genetics of HD

Found on human chromosome 4, the *HTT* gene contains a series of CAG repeats in the first exon. The repetitive trinucleotide sequence is translated into a series of glutamines in the HTT protein beginning with amino acid number 16. Every individual has CAG repeats in their *HTT* gene; most people have less than 35 repeats and no disease. In the HD population as a whole the number of repeats inversely correlates with disease symptoms; therefore the longer the repeat length the earlier the onset of disease. Most HD cases with adult onset have repeat sizes of 40-50 CAGs. For the individual however, the correlation between repeat length and diseases symptoms is loose with only 60% of the variance in HD age of onset can be accounted for by repeat length (Wexler et al. 2004). It is important to note that most HD models, including the ST*Hdh*$^{Q111/Q111}$ cellular model and YAC128 model used in this study, have a longer repeat length consistent with juvenile cases of HD. The large expansions are necessary to observe disease phenotypes in the mouse in a reasonable and testable period (Wheeler et al.

2002). Juvenile HD has a more severe disease progression with early degeneration occurring in the cerebellum.

HD is part of a family of nine distinct polyglutamine disorders; each of these disorders shares the phenomenon of genetic anticipation. Anticipation is defined as the reduction in age of onset across generations due to elongation of the repeat length with each subsequent generation. One group has indicated that the repeat instability is caused by DNA repair machinery that cannot function properly due to aberrant interactions between the polyglutamines and repair proteins such as CBP (Jung et al. 2007).

Genetic and Environmental Modifiers of Disease Progression

Huntington's patients all carry at least one mutated allele. Each child has a 50% chance of inheriting the mutation statistically, if the disease followed a simple Mendelian inheritance pattern. Indeed HD is passed down genetically as a true dominate disorder with larger expansion sizes. Interestingly, data suggests the mutated *HTT* allele is not passed with equal transmission rate with same-sex transmission rates being greater than different-sex transmission (i.e. Father to son and mother to daughter rates are greater than father to daughter rates). The same study also reported a tendency for same-sex siblings to have higher transmission rates. Sister sibling pairs had the highest correlation rates in the study but these were not significant (Wexler et al. 2004).

It is well established that the length of the CAG expansion affects the symptoms of the disease for the HD population as a whole. However, the association between the severity of HD symptoms and repeat length does not always correspond in the individual.

Additionally, multiple studies have found that the symptoms can be modified in patients and animal models of the disease (Georgiou et al. 1999; Gomez-Esteban et al. 2007; Panas et al. 2008; van Dellen et al. 2008). In a long term study of a large Venezuelan kindred, individuals with a repeat length between 40-50 CAGs the length only accounts for 44% of the variance in age of onset (Wexler et al. 2004). Due to the close relationship of these individuals, many of the genetic modifiers of HD would be common throughout the study population. Additionally, these patients would be exposed to common environmental agents that may modify disease. Therefore, the modifying factors are still undefined but both genetic and environmental factors are implicated.

Studies of homozygotic twins are limited in number but have shown the clinical presentation of the disease can be variable even though the repeat length is the same (Georgiou et al. 1999; Anca et al. 2004). Multiple twin studies have implicated other genetic modifiers and indeed other groups have found a high degree of inheritability (h=0.56) for the remaining variance after taking into account the repeat length (Li et al. 2003). A potential genetic interaction between HTT and the glutamate receptor gene has been examined by multiple groups. Specifically, modifications in the 3' untranslated region of *GRIK2* encoding the GluR6 subunit of the kainate receptor decrease the age of onset (Rubinsztein et al. 1997; MacDonald et al. 1999; Zeng et al. 2006). A follow up study in the Venezuelan kindred did not find an association between age of onset and *GRIK2* but the authors state that the SNPs in question are rare in the kindred (Andresen et al. 2007). However, genetic polymorphisms within the NR2A and NR2B subunits of the glutamate receptor where found to alter the age of onset in the Venezuelan study population (Andresen et al. 2007).

Because of the unique nature of the Venezuelan study, Wexler et al was able to analyze the total environmental effect between sibling groups. They report that 63% of the variance in age of onset that is not attributed to the repeat length was environmental (Wexler et al. 2004). The Venezuelans' average age of onset occurs at a significantly earlier age than other HD populations even without differences in average life expectancy of the population at large. The study hypothesized that the age of onset differences could be due to the living environment of the Venezuelan kindred. Most of the kindred depend on a nearby lake for survival. Lake Maracaibo is reported to be contaminated by both industry and sanitation issues (Wexler et al. 2004). Again, emphasizing a potential for environmental influence.

Environmental factors such as exercise and environmental enrichment have been examined in HD progression. Both exercise and enrichment have lead to improvements of HD symptoms in HD mouse models (Hockly et al. 2002; Lazic et al. 2006; van Dellen et al. 2008). The enrichment and exercise studies alter brain derived neurotrophic factor (BDNF) in these HD models (Zajac et al. 2009). Changes in BDNF led to increased memory and learning, as well as neurogenesis (Bartrup et al. 1997); (Barde 1994; Lee et al. 2002). Dietary changes as well are linked to improvements in HD symptoms through BDNF changes (Duan et al. 2003).

A Loss of Function or Gain of a Novel Function Mutation

The function of wild-type *HTT* is essential for survival and proper development; initiating one of the great debates in the HD field: Does the polyglutamine expansion cause HD via a gain of function or a loss of function? The answer to this very important

question would have profound effects on HD treatment strategies. If wild-type HTT function is lost in the presence of the mutation, treatments might focus on the downstream activities of the protein. Alternatively, if mutant HTT has gained a novel function, then that specific activity would need to be targeted for rescue by a potential treatment.

The loss of function argument leads to the hypothesis that knockout animals would be born and develop the disease as if they carried the mutation. Unfortunately, it is not that simple, as knockout animals die early in embryonic development (Duyao et al. 1995; Zeitlin et al. 1995; Lumsden et al. 2007). In fact, the lethal phenotype can be overcome by expression of the mutant protein (Dragatsis et al. 1998). Therefore, the embryonic lethality is likely due to a function of HTT that is secondary to the function that causes disease. Wild-type HTT does play a role in cell survival after embryogenesis (Van Raamsdonk et al. 2005; Leavitt et al. 2006; Van Raamsdonk et al. 2006)If the wild-type gene is deleted in post-mitotic cells, the mice show a progressive cell loss in the forebrain, giving credence to the loss of function hypothesis (Dragatsis et al. 2000). HD, however, is a dominant disorder, meaning that one mutant allele is sufficient to acquire the disease, indicating gain of function (Rubinsztein et al. 2003). The overexpression of wild-type HTT does have the ability to block cell death associated with the polyglutamine expansion in cellular HD models and mouse testes (Ho et al. 2001; Leavitt et al. 2001). In the striatum of the YAC mouse model, neuronal pathology can be reduced by overexpression of wild-type HTT but this slight improvement does not improve motor symptoms (Van Raamsdonk et al. 2006). Wild-type HTT is known to have antiapoptotic effects and therefore improvements in cell viability may be due to

inhibition of cell death pathways and not overcoming the effects of mutant HTT (Rigamonti et al. 2000; Leavitt et al. 2006). Alternatively, the mutant HTT may have a dominant negative effect, blocking the function of the wild-type protein. Taken together these studies have bolstered both sides of this debate, leading to the idea that the disease may be due to both a loss of normal wild-type function and a novel gain of function in the presence of the enlarged protein.

Molecular Causes of HD Neuropathology

Many molecular pathways and processes have been implicated in the progression of HD. Unfortunately, it is still unclear which, if any of these altered cellular mechanisms is the initial insult downstream of the polyglutamine expansion. Protein aggregation, mitochondrial dysfunction, excitotoxicity, loss of trophic support, and altered transcriptional regulation are some of the proposed molecular sources of the neuronal loss. To add to the complexity, malfunction in many of these cellular processes has been demonstrated to lead to similar alterations in cell health. For example, both mitochondrial dysfunction and excitotoxicity can lead to increases in oxidative stress, making the identification of the initial molecular insult challenging.

One of the hallmarks of HD is the formation of protein aggregates containing the polyglutamine expanded protein (Ross et al. 2004). With the protein aggregation appearing in the aforementioned affected brain regions and the strong correlation of disease severity with repeat length, aggregates were originally thought to be the primary cause of pathology (Vonsattel et al. 1985; Davies et al. 1997; Martindale et al. 1998;

Scherzinger et al. 1999). Recent studies have produced strong evidence that aggregation does not correlate with cell death and may actually improve neuronal survival (Saudou et al. 1998; Kim et al. 1999; Slow et al. 2005). HTT aggregates form throughout the brain of the *shortstop* mouse model of HD but there is no striatal cell loss or dysfunction at one year of age. The lack of neuronal loss in the presence of a heavy aggregate load indicates that HTT inclusions are not toxic (Slow et al. 2005). Importantly, in vitro studies have shown that blocking aggregation does not alter cell survival (Kim et al. 1999). Other studies have examined the aggregate load in specific cell types and shown that the cells that contain the most aggregates are, in fact, the surviving interneurons (Kuemmerle et al. 1999; Arrasate et al. 2004).

Initially, the HTT protein was proposed to alter mitochondrial function based on data from patients where multiple studies found altered metabolism in these individuals. The studies reported weight loss, reductions in N-acetylaspartate, elevated lactate, and altered glucose metabolism (Kuwert et al. 1993; Koroshetz et al. 1997; Clark 1998; Jenkins et al. 1998; Sanchez-Pernaute et al. 1999; Djousse et al. 2002; Mahant et al. 2003). The majority of these studies use brain images examining various markers of metabolism and mitochondrial function. Unfortunately, it is challenging to interpret these findings due to the prior and ongoing cell loss, which occurs with the disease. Perhaps the altered metabolism markers are due to the reduction in cell number. Animal studies have also reported weight loss and altered metabolism in the context of the polyglutamine expansion further indicating the role of HTT in nonmotor symptoms (Hurlbert et al. 1999; Jenkins et al. 2005; Dai et al. 2009). Importantly, many HD patients also suffer from diabetes, which is due to a lack of insulin secretion and altered

Akt activity (Ye et al. 2009). The insulin pathway is tightly tied to ATP production and mitochondrial function has been linked to diabetes (Johannsen et al. 2009).

Some studies have report that mitochondrial respiration may be reduced in HD models. Reduced activity in HD-affected brain areas and several peripheral tissues has been reported for complex I, II, and IV (Gu et al. 1996; Browne et al. 1997; Ciammola et al. 2006). This topic is controversial; indeed, the dysfunction may simply be due to the reduction in cell number compared to controls. An additional study reports that the mitochondrial complexes function efficiently but display decreased energy production due to a lack of substrates (Milakovic et al. 2005). Other groups report no alterations in mitochondrial respiration at all but these finding were in a pre-symptomatic HD model (Guidetti et al. 2001). This data indicates, that mitochondrial dysfunction may be downstream of another HTT insult. The variations in these studies may be due to the variety of cell types examined or the stage of the disease. The defects in mitochondrial energy metabolism may be specific to the striatum. Several studies have revealed mitochondrial dysfunction in the caudate and putamen but these finding where after atrophy of these regions and therefore cell loss may contribute significantly to these findings (Butterworth et al. 1985; Tabrizi et al. 1999; Guidetti et al. 2001; Sorolla et al. 2008).

The first animal models of HD that displayed the selective degeneration of the striatum were created by use of 3-nitroproprionic acid (3NPA), a mitochondrial complex II inhibitor (Beal et al. 1993; Brouillet et al. 1993; Brouillet et al. 2005). In primates, 3NPA-exposed animals present with many of the HD symptoms: dyskinetic movements and dystonic postures, as well as the striatal selective lesions (Beal et al. 1993; Brouillet

et al. 1995). The inhibition of succinate dehydrogenase by 3NPA has also been shown to increase cell death in cellular models of HD due to mitochondrial depolarization (Gines et al. 2003; Ruan et al. 2004). 3NPA stimulates calcium release (Rosenstock et al. 2004) and may explain why cellular models of HD show an increased vulnerability to 3NPA toxicity, leading to the hypothesis that HD alters calcium handling by the mitochondria (Trettel et al. 2000; Gines et al. 2003). Altered calcium homeostasis is also reported in mutant knock-in animals due to heightened instability of the mitochondrial transition pore (Choo et al. 2004).

Mitochondrial defects in calcium handling are also thought to play a role in increased excitotoxicity due to the HD mutation. Activation of N-methyl-D-aspartate receptor (NMDAR) in HD mouse model leads to an increase in apoptosis which can be blocked by inhibition of the mitochondrial transition pore (Tang et al. 2005). In the presence of the mutation, stimulation of NMDARs leads to an increased current and intracellular calcium levels (Zeron et al. 2004; Shehadeh et al. 2006; Fernandes et al. 2007).

Finally, mitochondria dysfunction can increase reactive oxygen species (ROS) within a cell. Markers of oxidative stress have been found in post-mortem brain studies of HD patients, showing damage to proteins, DNA, and phospholipids (Goebel et al. 1978; Browne et al. 1997). 8-hydroxydeoxyguanosine measurements of DNA oxidative damage have even been correlated with disease progression. Symptoms first appear in the dorsal striatum and then move throughout the striatum as the disease progresses (Browne et al. 1997). HD mouse and cell line studies also implicate oxidative stress in disease progression (Bogdanov et al. 2001; Choo et al. 2005; Mao et al. 2006). However,

it is possible that the oxidative stress is not the primary cause of the cell loss, as many of the animal studies do not observe oxidative damage before significant cell loss (Choo *et al.* 2005).

As mentioned above, excitotoxicity can lead to mitochondrial dysfunction and the production of free radicals (Castilho et al. 1999; Nicholls et al. 1999; Kim et al. 2000; Cowan et al. 2006; Fernandes et al. 2007). It has also been directly linked to the degeneration in HD. The hypothesis that excitotoxicity is the primary insult in HD stems from the similarities between HD pathology and that caused by injection of glutamate agonists (Coyle et al. 1976; Schwarcz et al. 1984; Beal et al. 1986; Beal et al. 1991; Fan et al. 2007). Medium spiny neurons are the first cells lost in HD, and are the targets of glutamatergic inputs from both the cortex and the thalamus. Modulating the signals at these synapses can block cell death induced by 3NPA (Greene et al. 1993; Bogdanov et al. 1998). Studies of one glutamate receptor have shown that increased levels of the NMDAR NR2B subunits enhanced death due to HD toxicity (Young et al. 1988; Albin et al. 1990; Chen et al. 1999). The NR2B subunits are responsible for increased calcium influx and are highly expressed on the MSNs (Christie et al. 2000; Cepeda et al. 2001). Indeed, mutant HTT increases cellular death in the presence of the NR2B subunit (Zeron et al. 2001; Zeron et al. 2002).

Not only can NMDAR modulation alter the HD cell death but also increased expression of BDNF has been shown to attenuate HD excitotoxicity (Canals et al. 1998; Alberch et al. 2002). BDNF is essential for the survival of MSNs and is transported by HTT along microtubules (Zuccato et al. 2001; Gauthier et al. 2004; Colin et al. 2008). The direction of transport depends on phosphorylation of wild-type HTT at serine 421

(S421); phosphorylation leads to anterograde transport (Zala et al. 2008). In the absence of phosphorylation, wild-type HTT moves BDNF towards the cell body (Humbert et al. 2002; Colin et al. 2008). BDNF can activate many cell survival pathways within the cells, but Akt/Insulin signaling has been shown to be essential for survival in striatal cells expressing mutant HTT (Humbert et al. 2002). Additionally AKT can directly phosphorylate HTT (Pardo et al. 2006). BDNF is a known regulator of transcription and, importantly, transcriptional dysregulation has been implicated in HD neurodegeneration (Zuccato et al. 2001; Chiang et al. 2007; Zuccato et al. 2007). HTT regulates BDNF at the level of transcription by sequestering a transcriptional regulator REST/NRSE in the cytoplasm. The neuron-restrictive silencer element (REST/NRSE) is responsible for the activation of many other genes. Therefore, HTT's ability to alter transcription is not limited to BDNF (Cattaneo et al. 2005). When mutated, HTT can no longer keep REST/NRSE in the cytoplasm, halting transcription of BDNF and other genes (Zuccato et al. 2003; Zuccato et al. 2007).

Metals have also been implicated in the progression HD. In addition to altered Ca handling in the ST*Hdh*$^{Q111/Q111}$ cells, HD patients also display elevated levels of Fe and Cu the corpus striatum in both post mortem and FMRI studies (Dexter et al. 1991; Bartzokis et al. 2000). However, many of the mechanisms of metal toxicity are the same pathophysiological pathways implicated in the progression of HD (e.g. altered calcium homeostasis, protein aggregation, increased oxidative stress, and altered energy metabolism). Importantly, altered metal levels in patients are only the beginning of the links between HD and metals. Cu has been shown to bind HTT, decreasing the solubility of the protein (Fox et al. 2007). The *HTT* gene increases its expression in response to an

elevation in Fe levels (Hilditch-Maguire et al. 2000). Additionally, zebrafish studies have demonstrated that the embryonic lethality due to *HTT* inactivation in this animal model is due to the incorrect utilization of Fe stores (Lumsden et al. 2007). The interaction of HTT and metals has been largely limited to the studies mentioned above but other studies have emphasized the potential of metals to promote the aggregation of amyloidogenic proteins such as α-synuclein (Uversky et al. 2001). Intriguingly, a mutation of a key iron storage protein leads to the disease neuroferritinopathy. The disease has a phenotype very similar to HD and ultimately leads to an adult onset loss of the basal ganglia due to accumulation of Fe in the brain (Curtis et al. 2001).

The primary insult of HD is still not determined even though many good candidates exist as described above. Understanding the primary source of HD toxicity is crucial for blockade of the neurodegeneration and production of potential therapies without widespread side effects. One unique feature of HD is the ability to diagnosis patients before the onset of symptoms through genetic testing. For example, in PD well over half the neurons of the substantia nigra are lost before symptoms occur and diagnosis does not normally occur until after the onset of symptoms. Therefore, there is a greater possibility that the HD progressive cell loss can be prevented than in other neurodegenerative disorders. Many of the proposed mechanisms of neurodegeneration are intertwined, so it is difficult to determine which may be the initial insult. Understanding how different toxicants can alter the molecular pathways implicated in HD may lead us to the molecular source of the disease.

Overview of Specific Aims

Current evidence has shown that though HD is a genetic disorder, disease progression can be modified by other factors. For example, the severity and disease progression of HD can be altered by the environment enrichment in a mouse model of HD (van Dellen et al. 2008). In humans, two separate studies have shown that the length of the CAG expansion alone cannot explain the variability in severity and age of onset of HD symptoms (Anca et al. 2004; Wexler et al. 2004). The environmental agents that modify the onset, progression, or clinical presentation of the disease in patients remain unknown. Identification of potential gene-environment interactions of *HTT* is paramount to understand how they may modify HD pathology.

Interestingly, a number of metals (e.g., Fe, Cu, and Mn) are associated with the selective degeneration of specific nuclei of the basal ganglia and share pathophysiological similarities with HD (Lee et al. 2006; Perl et al. 2007). The basis for this selectivity is unclear, but both the propensity of these metals to accumulate in specific brain regions and the nature of their cellular toxicity (e.g. oxidative stress, excitotoxicity, toxic interactions with dopamine, etc.) likely contribute (Erikson et al. 2003; Tarohda et al. 2004). In fact, at least four established mechanisms of metal toxicity are implicated in HD neuropathology, including oxidative stress, calcium signaling, protein aggregation, and altered energy metabolism. Additionally, Mn and other metals have been shown to promote aggregation of prion precursor protein, which like HTT, is amyloidogenic. Determining the modifiers of HD may also help identify pathophysiological mechanisms of the disease.

We hypothesized that a toxicant sharing pathophysiological mechanisms with HD would exhibit disease-toxicant interactions and would reveal mechanisms of selective degeneration and toxicity. To test this hypothesis, we propose the following questions: 1) Do metals display a disease-toxicant interaction with the polyglutamine expansion disorder HD? 2) What is the nature of the gene-environment/disease-toxicant interaction between HD and the divalent metals Mn and Cd? 3) Does mutant HTT impair Mn uptake, limiting Mn toxicity?

Specific Aim 1: Exploration of a Gene-Environment Interactions between Polyglutamine Expansion Disorders and Metals.

We developed and preformed a novel screen for gene-environment interactions between HD and various metals. Utilizing the well-published HD cellular model, ST*Hdh*$^{Q111/Q111,}$ in comparison to the wild-type ST*Hdh*$^{Q7/Q7}$, we used cell survival as a measurement of interaction between HD and metals. We explored the ability of Mn to alter aggregation of Ataxin1, another polyglutamine disorder, by transfection into HEK293 cells.

Specific Aim 2: Describe the Gene-Environment Interactions between the Metals Mn and Cd with HD.

Here, we explored the nature of a Mn-specific interaction we had discovered with the HD mutant protein. To determine whether the interaction is specific to the ST*Hdh* cell line, we will use both primary cultures and transfection studies. Additionally, we transfected into cells a truncated form of the HTT protein, in order to determine if the

polyglutamine domain is required for the interaction. Finally, we examined various markers of pathways that might be involved in Mn or HD pathophysiology by assessing proteins by western blot.

Specific Aim 3: Test the Contribution of Mn Transport to the HD-Mn Interaction

We continued to explore the HTT-Mn interaction by additional analysis of pathway differences discovered in aim 2. In both the ST*Hdh* cellular model and a thoroughly studied HD animal model, we analyzed how the HD mutation might alter Mn-induced cell death, focusing on accumulation of the metal within the cell and brain.

CHAPTER II

DISCOVERY OF A NEUROPROTECTIVE GENE-ENVIRONMENT INTERACTION BETWEEN HUNTINGTIN AND MANGANESE

Introduction

In HD, degeneration of the medium spiny neurons of the corpus striatum occurs well prior to other affected brain regions such as the cortex (Imarisio et al. 2008). This selective degeneration occurs despite widespread expression of the disease-causing polyglutamine-expanded protein, HTT. Thus, other factors within the striatum may uniquely increase the vulnerability of this area to the pathophysiological mechanisms behind HD. Interestingly, the toxicant 3-nitroproprionic acid (3NPA), a mitochondrial complex II inhibitor, exhibits an HD-like striatal specific neurodegeneration (Beal et al. 1993). One explanation of this common pathology is that both mutant HTT and 3NPA might impinge upon a shared pathophysiological vulnerability inherent to the striatum. Indeed, both HD and 3NPA toxicity cause mitochondrial dysfunction, oxidative stress, excitotoxicity, and altered metal homeostasis (Dexter et al. 1991; Beal et al. 1993; Imarisio et al. 2008). In both the STHdh striatal cell model of HD and the R6/2 animal model, expression of polyglutamine expanded HTT increases vulnerability to the toxin 3NPA (Bogdanov et al. 1998; Ruan et al. 2004; Brouillet et al. 2005).

We postulate that toxicants acting upon pathophysiological targets modulated in HD will exhibit disease-toxicant interactions, even if patients are not normally exposed to these toxicants. Identifying these interactions may uncover mechanisms of selective neurodegeneration. To test this hypothesis, we designed a disease-toxicant interaction screen to examine alterations of cell viability due to various toxicants in the context of the HD mutation. Due to their diverse toxicology and similarities to HD pathology, we focused on neurotoxic metals (Gaeta et al. 2005). Several studies have also revealed elevated levels of Fe and Cu in the striatum of HD patients and animal models (Dexter et al. 1991; Fox et al. 2007). Furthermore, *HTT*, has been functionally linked to Fe homeostasis, and HTT is a Cu-binding protein (Trettel et al. 2000; Fox et al. 2007; Simmons et al. 2007). Thus, metal exposure is a reasonable starting point for a search of environmental factors with the potential to modulate HD pathophysiology.

Materials and Methods

Chemicals, Reagents, and Cell Culture Supplies

Cell culture media and supplements were obtained from Mediatech (Manassas, VA) unless indicated. Cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), L-glutamine, 400 μg/ml G418 and Penicillin-Streptomycin. Metals and toxicants used in survival assays were from Alfa Aesar (Ward Hill, MA) unless indicated: 3-nitroproprionic Acid (Sigma, St. Louis, MO), $FeCl_3$, (VWR, West Chester, PA), $MnCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $PbCl_2$, $NiSO_4$, and $ZnCl_2$. MTT Reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) salt (VWR, West Chester, PA), Sorenson's Buffer (0.1 M glycine, 0.1 M $NaCl_2$, pH 10.5), dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO).

The inorganic salt concentration is listed for the following metals: Ca, Mg, Fe, potassium, and sodium are in significant levels in the DMEM we use for cell culture. Based on the DMEM technical documents from Sigma Aldrich, the concentrations of sodium is ~167mM, calcium (Ca) 2.4mM , Fe 0.2 μM, Mg 811 μM, and potassium 5.4mM. Mn levels were not reported in the technical materials.

Description of ST*Hdh* Cellular Model of HD

The ST*Hdh* cell model of HD was created from a targeted knockin of the mouse *HTT* gene (Cattaneo et al. 1998; Trettel et al. 2000; Wheeler et al. 2000). The cell lines express full-length (wild-type or glutamine expanded) *HTT* from the endogenous locus allowing comparisons of phenotypes between wild-type (ST$Hdh^{Q7/Q7}$) and mutant (ST$Hdh^{Q111/Q111}$) cells. Homozygotes of mutant embryos expressing 111 CAG repeats or wild-type 7 CAG repeats were utilized to create this cell model by Trettel et al. Embryos were infected with a temperature sensitive SV40 large T antigen that forces cell division (Cattaneo et al. 1998; Trettel et al. 2000). At 33ºC, the permissive temperature where the viral SV40 construct is active, both cell lines can be maintained and passaged. Cell growth at 39ºC is inhibited (Cattaneo et al. 1998).

Cell Culture

The clonal striatal cell lines – both mutant ST$Hdh^{Q111/Q111}$ and wild-type ST$Hdh^{Q7/Q7}$ were a generous gift from Marcy Macdonald, PhD (Massachusetts General

Hospital. Human embryonic kidney (HEK) 293T cells can be purchased from ATCC. The cells were cultured in DMEM with 10% FBS and Penicillin-Streptomycin at 37 ºC.

Frequency of Cell Division

Mutant ST*Hdh*$^{Q111/Q111}$ and wild-type ST*Hdh*$^{Q7/Q7}$ cells were plated as for survival assays and cultured under standard conditions at 33 ºC. After 30 hours, cells were harvest and counted. Frequency of division was determined by the equation $N_t=N_o2^{tf}$. Where N_t= number of cells at harvest, N_o= number of cell plated, t= time in culture, and f= divison rate or frequency (Sherley et al. 1995).

Cell survival Assays

ST*Hdh*$^{Q111/Q111}$ and wild-type ST*Hdh*$^{Q7/Q7}$ cells were plated at equal density (350000 cells per 10 cm^2) the evening before treatment. Toxins or metals were added to the culture media the next morning, and cells exposed for 26 to 30 hours. Cell viability was assessed with a hemacytometer using trypan blue exclusion or MTT assay. Trypan blue exclusion: Cells were harvested after exposure as described above with trypsin and counted on a hemacytometer utilizing trypan blue exclusion. The number of viable cells relative to the untreated for each genotype is shown as percent untreated. MTT assay: After treatment as described above, the media was removed, and 500 µl of 0.5% MTT salt in MEM (Invitrogen, Carlsbad, CA) containing FBS and Penicillin-Streptomycin was added to each well for 4 hours. Then the media was removed and Sorenson's Buffer was diluted 2.5:20 in DMSO. 200 µl of Sorenson's Buffer in DMSO was added to the empty wells to dissolve the precipitate. The plates were returned to the incubator until all the

MTT salt precipitate could no longer be visualized under a microscope. 60 µl from each well was removed and placed in a 96-well plate. Absorbance at 590 nm was read on a Tecan (Durham, NC) plate reader (Ehrich et al. 2000). The average absorbance relative to the untreated control is graphed as percent untreated.

Colorimetric LDH Assays

We used the In Vitro Toxicology Assay Kit (TOX7) (Sigma, St. Louis, MO). ST*Hdh* cells were counted using a hemacytometer and immediately lysed according to the manufactor's protocol. Absorbance was measured at 490nm wavelength.

Analysis of Ataxin-1 Aggregates

HEK 293T cells were passaged onto glass coverslips and transfected with glutamine-expanded full-length Ataxin-1 (ATXN1[82Q]) (Bowman et al. 2005; Bowman et al. 2007). After expressing ATXN1[82Q] for 24 hours, cells were treated with various concentrations of Mn(II). Next, cells were harvested and fixed with 4% paraformaldehyde (diluted from 16% solution, Electron Microscopy Sciences, Hatfield, PA). Cells were permeablized with PBT (PBS with 0.1% Triton X-100 (Sigma, St. Louis, MO), stained for ATXN1[82Q] and coverslipped with ProLong Gold antifade reagent with DAPI nuclear stain (Invitrogen, Carlsbad, CA). Fraction of cells with inclusions (bright green puncta, see arrow heads) relative to number of transfected cells was determine by automated image analysis using NIH ImageJ software.

Statistical Analysis

Univariate ANOVA was performed using SPSS software (SPSS, Inc., Chicago, IL). All *post-hoc* analyses were done assuming equal variances, error bars are expressed as standard error of the mean (SEM). *Post-hoc* analyses were completed using Microsoft Excel (Redmond, WA). Standard student's t-tests were done for differences between groups.

RESULTS

Description of ST*Hdh* Cellular Model of HD

Previous publications have shown that the ST$Hdh^{Q111/Q111}$ cells have slower growth rates and lower expression of the mutant protein when compared to the wild-type Q7 line (Trettel et al. 2000). We have also seen a reduction in cell division rates in the presence of the HD mutation (Figure II.1.). ST$Hdh^{Q111/Q111}$ cells have a reduced frequency of cell division compared to wild-type ST$Hdh^{Q7/Q7}$.

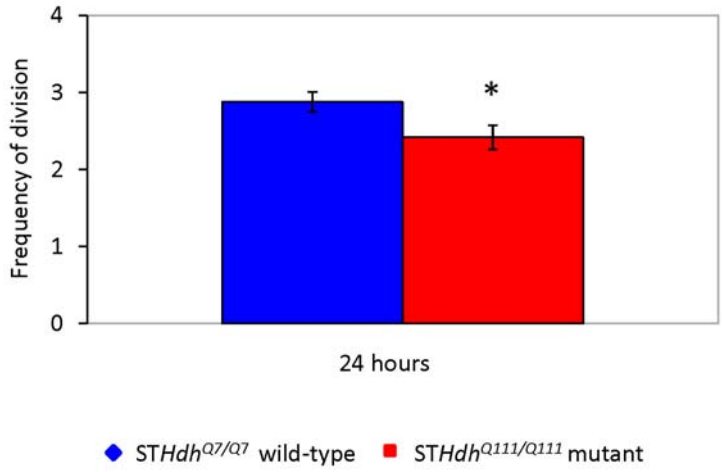


Figure II.1. ST*Hdh* Cell Growth Rates. Mutant (red) and wild-type (blue) cells were plated evenly and the number of cells by trypan blue exclusion. N=7 independent samples (± SEM). Significantly different in frequency of cell division (* p=0.05 *posthoc t-test*) between wild-type and mutant.

The ST*Hdh*$^{Q111/Q111}$ cell line have also been shown to have an impairment of the mitochondrial respiratory chain, altered calcium handling, sensitivity to the toxin 3NPA, and heightened excitotoxicity (Seong et al. 2005; Mao et al. 2006; Milakovic et al. 2006; Oliveira et al. 2006; Xifro et al. 2008). Each of these cellular phenotypes could affect our analysis of viability. One classic assay to examine cell viability is lactate dehydrogenase activity (LDH). LDH enzyme is released during cell death but functions in the cytoplasm of healthy cells to create energetic substrates for the mitochondria. Based upon differences in LDH activity without Mn exposure we report in chapter III, we examine the LDH activity in equal numbers of mutant or wild-type cells and discovered that the ST*Hdh*$^{Q111/Q111}$ cells have reduced LDH activity in the absence of any toxin (Figure II.2. and Figure III.2). Absolute differences are difficult to assess, however, the data from

these separate figures led us to use the MTT assay, which measures the activity of mitochondrial reductase as a measure of cell viability.

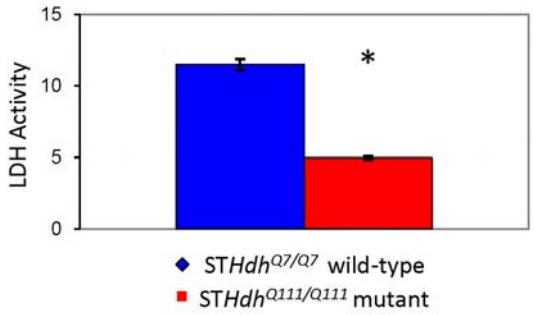
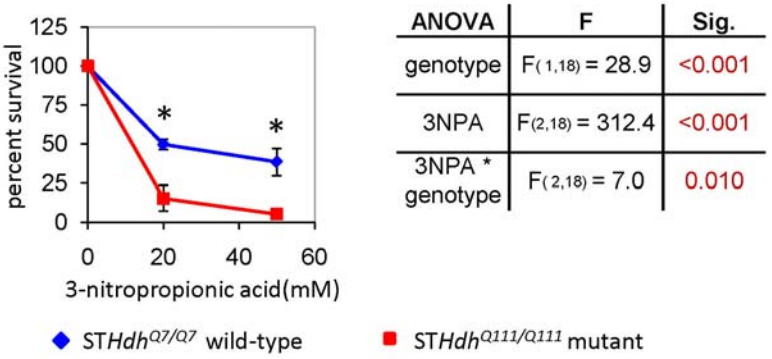


Figure II.2. LDH Activity in Wild-type and Mutant ST*Hdh* Cells. An equal number of cells (~7000) where harvest, lysed, and LDH activity was accessed. n=2 (±SEM). Significant differences in activity (* $p<0.05$ *posthoc* *t*-test) between wild-type and mutant.

A HD-Toxicant Interaction Screen

To establish an experimental model of gene-environment interactions between metals and mutant *HTT,* we utilized the HD mouse striatal cell line. We focused on cell survival as a basic toxicological phenotype for the screen. Previous research had shown that the mutant cell line (ST$Hdh^{Q111/Q111}$) line has increased sensitivity to the toxicant 3NPA (Ruan et al. 2004). Therefore, as a positive control, we examined cell survival in the cell model following 30 hours exposure to 3NPA, confirming that the mutant ST$Hdh^{Q111/Q111}$ line has a significant ($p<0.05$) decrease in cell survival relative to wild-type cells (Figure II.3.).



ANOVA	F	Sig.
genotype	$F_{(1,18)} = 28.9$	<0.001
3NPA	$F_{(2,18)} = 312.4$	<0.001
3NPA * genotype	$F_{(2,18)} = 7.0$	0.010

Figure II.3. Survival Curve for 3NPA Cytotoxicity in Wild-type versus HD Striatal Cell Lines. 350,000 cells of wild-type ST*Hdh*$^{Q7/Q7}$ (blue) or mutant ST*Hdh*$^{Q111/Q111}$ (red) cells were exposed to indicated amounts of 3NPA. The number of viable cells relative to the untreated control is shown as percent untreated (± SEM). Two-way univariate ANOVA showed a significant effect of genotype, exposure, and two-way interaction of genotype by exposure. Significant differences in survival (* $p<0.05$ *posthoc t*-test) between wild-type and mutant cells at specific exposure levels are shown, n=3.

After validating the HD striatal model for detection of gene-environment interactions relevant to HD, we initiated the screen to determine if expression of mutant *HTT* modulates sensitivity to a diverse set of metals. We screened 8 neurotoxic metal ions (Fe, Cu, Pb, Co, Zn, Ni, Cd, and Mn) by generating concentration response curves (Figure II.4.). For each metal, preliminary experiments were carried out to identify concentration ranges with low to high degrees of cytotoxicity. Then, concentrations were chosen to generate a survival curve to span concentrations from non-toxic to highly toxic exposures. We were unable to induce a high degree of cell death (>40%) for Fe(III) and Pb(II) to due to insufficient solubility at higher concentrations. Nevertheless, statistical analysis of cell survival by two-way ANOVA revealed a significant effect of exposure on cell survival for each of the eight metals ($p<0.001$). Cell survival assessment for six of the eight metals tested, Fe(III), Cu(II), Pb(II), Co(II), Zn(II), and Ni(II), produced indistinguishable survival curves between wild-type and mutant STHdh cells (Figure

II.4.). In contrast, the survival curves for Cd(II) and Mn(II) both showed differences in survival between wild-type and mutant cells (Figure II.4.). The survival curve for Cd(II) showed an increased sensitivity of the mutant ST*Hdh*$^{Q111/Q111}$ cells to cadmium (Cd) cytotoxicity. Statistical analysis by two-way univariate ANOVA showed a significant effect of genotype ($F_{(1,29)}$=5.31, p=0.029) on cell survival. *Posthoc* analysis indicated that the wild-type line had significantly ($p<0.05$) higher survival at 50μM Cd(II) relative to mutant cells (Figure II.4.). Analysis of Mn toxicity in the ST*Hdh* cells revealed an unexpected gene-environment interaction, in which cells expressing mutant *HTT* were resistant to Mn toxicity. Statistical analysis by two-way repeated measures ANOVA found a significant effect of genotype ($F_{(1,8)}$=323.2, $p<0.001$) on cell survival. *Posthoc* analysis indicated that the mutant line had significantly ($p<0.01$) higher survival at 50μM, 100 μM and 300 μM Mn(II) relative to wild-type cells (Figure II.4.).

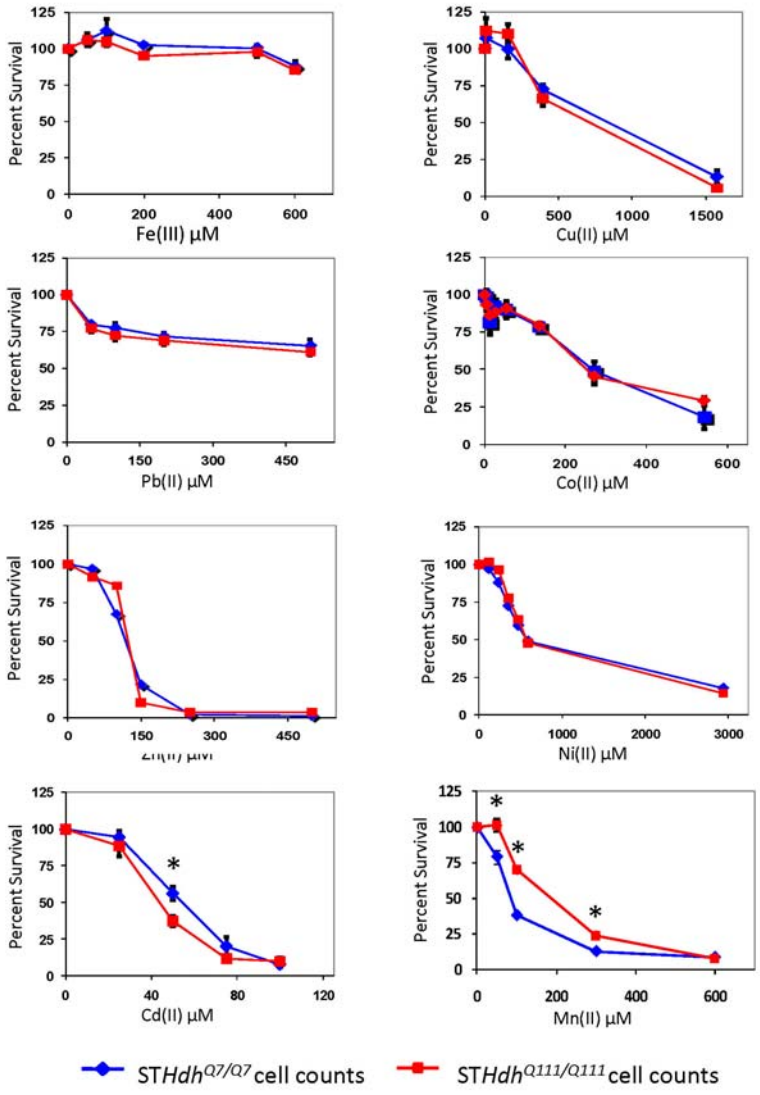
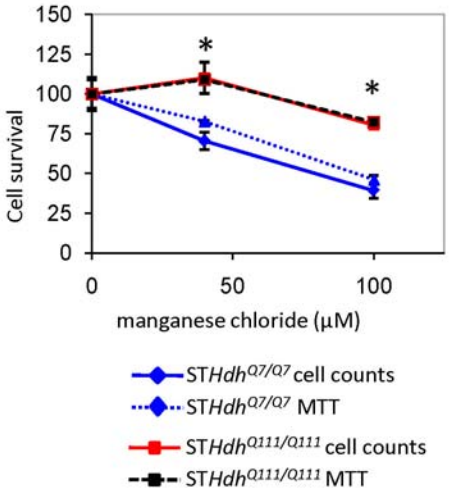


Figure II.4. HD-Metal Toxicity Cell Survival Screen. Wild-type ST*Hdh*$^{Q7/Q7}$ (blue) or mutant ST*Hdh*$^{Q111/Q111}$ (red) cells cell survival was assessed by MTT assay except for Cu, which was assessed by trypan blue exclusion. The average absorbance (or mean cell counts for Cu) relative to the untreated control for each genotype is plotted as percent cell survival (± SEM). n=number of experiments with between 3 and 6 independent replicate wells per experiment. Cu(II) (n=4), Fe(III) (n=3), Cd(II) (n=4), Zn(II) (n=3), Pb(II) (n=3), Co(II) (n=4), Ni(II) (n=3), and Mn(II) (n=2). ANOVA showed a significant effect of exposure for all metals ($p<0.001$), and a significant difference between genotypes only for Cd(II) ($p=0.029$) and Mn(II) ($p=0.001$). Significant differences in survival (* $p<0.05$ *post-hoc t*-test) between wild-type and mutant cells at specific exposure levels are shown.

Mn survival curve

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay used in the initial screen is not a direct measure of cell survival. This assay measures mitochondrial reductase activity, and can also indicate mitochondrial stress. Therefore, to confirm directly that the HD-Mn interaction involved changes in cell survival we utilized hemacytometer counts with trypan blue exclusion paired with MTT from replicate wells. These data demonstrate that MTT changes directly correlate with the change in cell number (Figure II.5.). Additionally, we used trypan blue exclusion to generate new expanded survival curves (Figure II.6.). The expanded hemacytometer-based survival curve for Mn confirmed the gene-environment interaction seen by MTT assay (Figure II.4. versus Figure II.6.). Statistical analysis by two-way univariate ANOVA found a significant effect of genotype ($F_{(1,88)}$=59.3, $p<0.001$) on cell survival with Mn exposure, in addition a two-way interaction between genotype and Mn exposure was detected ($F_{(10,88)}$=2.1, $p=0.039$). Over a broad range of Mn exposures (40-300 μM) the HD mutation limited Mn cytotoxicity of the metal when compared to the wild-type cell line ($p<0.05$). Furthermore, the expanded curve revealed that unlike the wild-type ST$Hdh^{Q7/Q7}$cells, which have a typical monophasic cytotoxic response, mutant ST$Hdh^{Q111/Q111}$ cells display a biphasic response to Mn exposure. At the highest concentrations of Mn tested (400-500 μM) the toxicity is not significantly different between wild-type and mutant cell lines. Therefore, the survival curve for Mn(II) shows a gene-environment interaction wherein Mn counters an inherent decreased viability of the mutant *HTT* cells, while mutant *HTT* counters the toxic effects of Mn exposure. A

student t-test of cell survival data comparing cells exposed to 40 μM Mn with unexposed cells within each genotype indicates a significant increase in viability only in the mutant cells (Figure II.7.).



ANOVA	F	Sig.
genotype	$F_{(1,18)}$=3.41	<0.001
Mn	$F_{(2,18)}$=5.78	<0.001
method	$F_{(1,18)}$=0.001	0.052
genotype * Mn	$F_{(2,18)}$=4.61	<0.001
genotype * method	$F_{(1,18)}$=0.56	0.952
Mn * method	$F_{(2,18)}$=1.00	0.530
genotype * Mn * method	$F_{(2,18)}$=0.36	0.868

Figure II.5. Mn Toxicity in Striatal Cells is Detected Equally by Independent Cell Survival Assays. Equal numbers of wild-type ST*Hdh*$^{Q7/Q7}$ (blue) or mutant ST*Hdh*$^{Q111/Q111}$ (red) cells were plated, and exposure to indicated amount of manganese chloride. (± SEM of 2 independent wells for hemacytometer counts and 3 independent wells for MTT assay). Tables show statistical analysis from a univariate ANOVA for each curve. Significant effects of genotype, exposure, or two-way interaction of genotype by exposure are designated by red numbering. There is no significant effect of method (blue numbering) on either genotype, Mn-exposure, or genotype by Mn-exposure interactions. * Significant difference in survival ($p<0.05$ *post-hoc t*-test assuming unequal variance) between wild-type and mutant cells at indicated exposures.

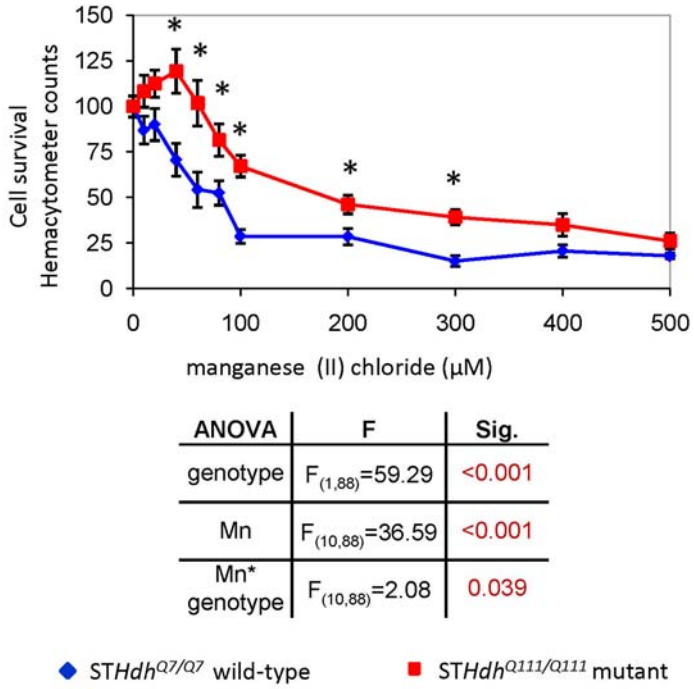


Figure II.6. Concentration-Response Curve for Mn Shows a Gene-Environment Interaction with HD. The number of viable cells, wild-type STHdh$^{Q7/Q7}$ (blue) or mutant ST*Hdh*$^{Q111/Q111}$ (red), is graphed relative to the untreated control is shown as percent untreated (± SEM. n=3 independent experiments). Tables show statistical analysis from a two-way univariate ANOVA for each curve. Significant effects of genotype, exposure, or two-way interaction of genotype by exposure are designated by red numbering. * Significant difference in survival ($p<0.05$ *post-hoc t*-test assuming unequal variance) between wild-type and mutant cells at indicated exposures.

Mn Induced Aggregation in a Polyglutamine Disease Model

Polyglutamine expanded HTT aggregates in animal models and human patients, but the role of aggregation of HTT in disease progression is complicated. Unfortunately, the full-length protein does not readily form microscopically visible inclusions in cell culture models. The process of amyloidogenesis or aggregate formation is believed to be similar between all polyglutamine disorders (Thakur et al. 2002). Aggregation has been shown recently to correlate inversely with cellular dysfunction and death in

spinocerebellar ataxia type 7 and type 1 (Bowman et al. 2005; Bowman et al. 2007). Knowing that Mn can increase the aggregation of prion precursor protein, we wanted to test the ability of Mn to alter aggregation of a polyglutamine disorder to understand how Mn may alter cell survival. Therefore, we chose a cellular model of spinocerebellar ataxin type 1 containing CAG a repeat length of 82 (ATXN1[82]). We chose ATXN1[82] because of its known ability to aggregate in cellular models (Bowman et al. 2007). HEK293T cells, a non-neuronal cell line, were transfected with ATXN1[82] and treated with 250 or 500 µM $MnCl_2$. We report that 500 µM $MnCl_2$ significantly increases the rate of aggregate of ATXN1[82] above unexposed ATXN1[82] expressing cells (Figure 11.8.).

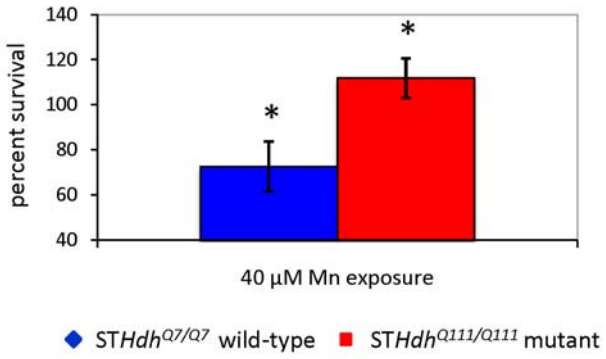


Figure II.7. ST*Hdh*$^{Q111/Q111}$ Cell Line Survival is Increased in the Presence of 40µM Mn. Viability of wild-type STHdh$^{Q7/Q7}$ (blue) or mutant ST*Hdh*$^{Q111/Q111}$ (red) 30 hours after exposure to 40µM Mn(II) chloride. The number of viable cells relative to the untreated control is shown as percent untreated (± 95% confidence intervals. n=12 independent experiments). * Significant difference in survival when compared to untreated ($p<0.05$ *t*-test assuming unequal variance).

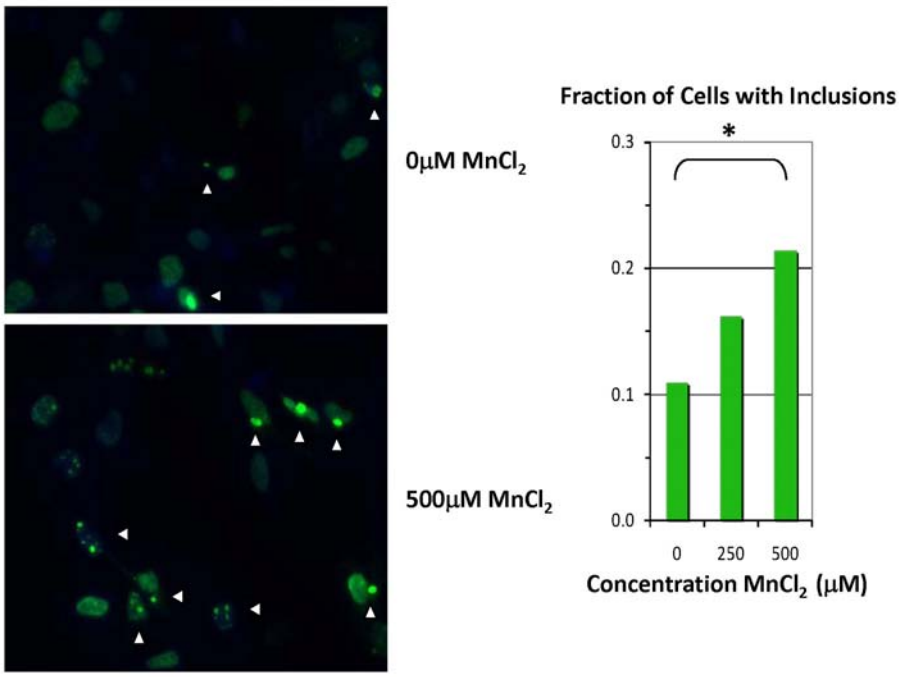


Figure II.8. Increased Aggregates of SCA1[82Q] in the Presence of Mn. HEK293 cells were plated, transfected with ATXN1[82Q] before exposure to media supplemented with indicated amounts of Mn(II) chloride. After 3 days exposure to Mn, cells were probed by immunofluorescence for ATXN1[82Q] (green) and DAPI (blue). Fraction of cells with inclusions (bright green puncta, see arrow heads) relative to number of transfected cells was determine by automated image analysis using NIH ImageJ software. The mean fraction of cells with inclusions (± SEM) is plotted. * Significant difference in survival ($p<0.05$ *t*-test assuming unequal variance) compared to exposed cells.

Conclusions

We used a disease-toxicant interaction screen to evaluate toxicants that may share pathophysiological mechanisms underlying HD neurodegenerative processes. The approach exploited the diverse toxicology of metals to reveal processes that may underlie gene-environment interactions in disease. Metals were the toxicant of choice for this screen because they are known to alter cellular pathways also implicated in HD pathology. We tested eight metals and the mitochondrial complex II inhibitor 3NPA for modification of cell survival in the presence of either wild-type HTT or a polyglutamine-

expanded form of the protein. 3NPA was a positive control showing increased toxicity in the presence of the glutamine-expansion as previously reported (Figure II.3.). The wild-type and HD mutant cell lines showed no difference in their response to the metals Cu(II), Fe(III), Pb(II), Co(II), Ni(II), or Zn(II) (Figure II.4.). In contrast, the mutant HD cell line was found to have enhanced sensitivity to Cd(II) toxicity, and resistance to Mn(II) toxicity. The selective effect of these two metals strongly suggests that the HD mutation can alter the influence of specific environmental toxicants on striatal cell lines and validates the utility of a disease-toxicant interaction screen to identify pathways of gene-environment interactions.

Oxidative stress is known to be a key factor in Cu and Fe mediated toxicities and is a potential mechanism of HD pathology. The lack of a significant difference in toxicity between genotypes with these metals is consistent with an earlier study that found no difference in vulnerability to oxidative stress in another HD cell model (Figure II.4.) (Snider et al. 2003). Furthermore, it suggests that a change in sensitivity to oxidative stress is not the source of the altered vulnerability of mutant ST*Hdh*$^{Q111/Q111}$ cells to Cd, Mn, or 3NPA (Figure II.3. and II.4.).

Additionally, we report a significant increase in the formation of microscopically visible polyglutamine inclusions in a cellular model of spinocerebellar ataxin type1 but only at a high concentration of Mn exposure (500 μM) (Figure II.8.). Although, Mn can alter microscopically visible aggregates, recent evidence in the HD field have implicated the soluble aggregates, which are not microscopically visible, as the pathological form (Luo et al. 2008). The role of large microscopically visible, insoluble aggregates may be to increase survival by sequestering the toxic species within the cell (Williams et al.

2008; Sathasivam et al. 2009). Importantly, we needed high concentrations of Mn needed to induce a significant effect on aggregation. These Mn levels are well above the concentrations where we see the differences in survival between ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q111/Q111}$ (Figures II.4, 5, and 6). Therefore, the influence on Mn on aggregation does not appear to be the same mechanism by which Mn is influencing cell survival in the mutant cells. Further work is necessary to understand what role aggregation may play in survival.

The gene-environment interaction identified here must be further characterized to understand the impact on cellular pathology. To characterize this interaction, first we must confirm the interaction in an additional model system. The ST*Hdh* cell line is a well-defined model of HD, which contains many of the hallmark phenotypes of the disease seen in both animal models and humans (Trettel et al. 2000; Gines et al. 2003; Gines et al. 2003; Seong et al. 2005; Mao et al. 2006; Milakovic et al. 2006; Oliveira et al. 2006; Xifro et al. 2008). These cells, however, are known to have different expression rates of HTT and that may have a profound effect on survival given that wild-type HTT has neuroprotective properties (Trettel et al. 2000). Additionally, the slower cell division rates in the mutant line may influence the uptake and toxicity of Mn (Figure II.1).

Interestingly, a decrease LDH activity has been reported in the R6/2 mouse model of HD (Figure II.2. and III.2.) (Fox et al. 2007). Microarray of the ST*Hdh* cell line show altered expression of this enzyme implicating enzyme dysfunction (Lee et al. 2007). Not only do these cells have a reduction in cell division rates but also LDH levels are lower in the mutants compared to wild-type (Figure II.1. and 2.).

Mn studies have shown that there are dose dependent effects of Mn on the mitochondrial electron transport chain. At low levels (10 μM), Mn stimulates the electron transport chain; however, it becomes inhibitory at higher concentrations mimicking our Mn response curve in the ST*Hdh*$^{Q111/Q111}$ cells (Heron et al. 2001). Altered Mn homeostasis could influence the activity of this enzyme directly or indirectly by altering other metals in the brain such as Cu, that alters LDH activity (Pamp et al. 2005). Therefore, the different LDH activities we have seen in this HD model though intriguing complicate our ability to use this as a simple marker of cell survival. Given that the *in vivo* toxicity of HTT is due to the expanded polyglutamine domain, we must determine if the polyglutamine domain is required for this novel phenotype. Finally, close examination of the cell signaling pathways known to be altered by HD or Mn could help identify the mechanisms involved in this phenotype and potential mechanisms of disease.

CHAPTER III

CHARACTERIZATION OF HUNTINGTIN AND MANGANESE GENE-ENVIRONMENT INTERACTION

Introduction

We utilized the screen to uncover interactions based on the hypothesis that a toxicant sharing pathophysiological mechanisms with HD would exhibit disease-toxicant interactions and would reveal mechanisms of selective degeneration and toxicity. We have discovered a novel gene-environment interaction between HTT and the divalent metals Mn and Cd in our previous results (Figures II.4.-II.7.). Mutant cells have increased sensitivity to Cd (Figure II.4.). However, cells that express the HD mutation display reduced toxicity to Mn when compared to cells expressing wild-type HTT. The interaction is specific to the metal Mn and does not occur with the other metals that we tested (Cu. Co, Cd, Zn, Pb, Ni, and Fe) (Figure II.4.). The ability of the HD mutation to block toxicity to Mn is one of few results where polyglutamine-expanded HTT improves cell viability.

Common pathophysiological similarities exist between HD and Mn toxicity. Mn accumulates in the basal ganglia and overexposure to Mn^{2+} is associated with selective neurodegeneration of this brain region, the same region altered by HD (Madsen et al. 2007; Peng et al. 2007; Perl et al. 2007; Stanwood et al. 2009). The reason for regional

specificity in not understood but the involvement of multiple cellular pathways has been shown. A few of the potential mechanisms of death common to both HD and Mn toxicity are oxidative stress, calcium signaling, protein aggregation and altered energy metabolism. (Erikson et al. 2003; Tarohda et al. 2004).

Data indicate that wild-type HTT has a pro-survival effect in the cell; first indicated by the embryonic lethality of *HTT* knockout mice (Duyao et al. 1995; Nasir et al. 1995; Zeitlin et al. 1995). Additional studies in a *Drosophila* model found neurodegeneration after RNAi knockdown of the HTT protein and a mouse model shows cell loss in the mature brain with inactivation of *HTT* (Dragatsis et al. 2000; Gunawardena et al. 2003). HTT is known to alter multiple pathways that may increase cell survival. For example the wild-type protein increases BDNF, an essential neurotrophic factor, transport (Zuccato et al. 2001; Pineda et al. 2005; Zuccato et al. 2005; Gharami et al. 2008; Zala et al. 2008). It also blocks caspase 3 activity, a protein that leads to cell apoptosis (Zhang et al. 2006). These cell survival pathways converge on the activity of protein Akt, specifically increased phosphorylation at S473 is associated with neuroprotection (Brunet et al. 2001). In the ST*Hdh* cell model, Akt phosphorylation at S473 is reported to be elevated in the mutant ST$Hdh^{Q111/Q111}$ line, with no change in the levels of total Akt between mutant and wild-type cells (Gines et al. 2003). Previous research has shown that exposure to Mn(II) is associated with activation of the Akt cell stress pathway via phosphorylation of S473 (Bae et al. 2006; Liao et al. 2007). We hypothesize that the gene-environment interaction between HTT and Mn will be shown in pathways common to both toxicities. Additionally, analyses of these common

signaling mechanisms will allow us to describe the disease-toxicant interaction more thoroughly.

Methods

Chemicals, Reagents, and Cell Culture Supplies

Cell culture reagents are from Mediatech (Manassas, VA) unless indicated. Cell lines were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), L-glutamine, and Penicillin-Streptomycin. 400 μg/ml G418 was added to media to select for expression of wild-type or mutant *HTT* ST*Hdh* cell lines. Metals, Mn(II) chloride, and Cd(II) chloride, used for assays were from Alfa Aesar (Ward Hill, MA). Buffers and solutions for assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt (VWR, West Chester, PA), Sorenson's Buffer (0.1 M glycine, 0.1 M $NaCl_2$, pH 10.5), dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). PBT (PBS with 0.1% Triton X-100 (Sigma, St. Louis, MO), and 4% paraformaldehyde in PBS (diluted from 16% solution, Electron Microscopy Sciences, Hatfield, PA) were used for cell imaging studies.

Antibodies

Human HTT (2166) 1:20000 (Millipore, Billerica, MA), Pan Akt 1:1000 (Cell Signaling, Beverly, MA), phospho-Akt 1:1000 (Cell Signaling, Beverly, MA), and actin

1:2000 (Developmental Studies Hybridoma Bank, Iowa City, IA). Appropriate secondary antibodies from Jackson Immunoresearch Laboratories (West Grove, PA) were used at 1:15000 accordingly: IgG mouse, IgG rabbit, and IgM mouse.

Cloning of Full HD constructs

Full length *HD* constructs were made for both polyglutamine and wild-type *HTT*. Sequence alignments utilize the NCBI session number NM_002111. The construct was made in 3 main pieces from a *HD* construct in pUAST, a gift from Juan Botas (Baylor College of Medicine): the 5' pieces were assembled by a digest and a three step PCR protocol, the middle and largest fragment was created by a simple 2 step subcloning procedure and the 3' end was made using a PCR reaction to create new restriction sites. *The 5' end:* From the pUAST vector containing *HD*, we subcloned using a NotI site in the vector upstream of the ATG start of *HD* and an endogenous XbaI site (bp 3782). The polyglutamine expansion of 111 repeats was created by PCR from the YAC128 mouse model. PCR primers were designed with Not1 site upstream of the transcriptional start and RsrII site below the polyglutamine expansion (bp384). PCR reaction 1:5' oligonulceotide ATATGCGGCCGCTCAGGTTCTG; 3' oligonulceotide TCAGCCACA GCCGGTCCGGGTGGCG. A seconded PCR was done with primers containing the RsrII site at the five prime end. PCR reaction 2: 5' primer CGCCACCCGGACCGGCT GTGGCTGA; 3' primer CTCCGAGGGGCACCATTC. The 3' oligonulceotide was designed to overlap the endogenous Xho site (bp655). PCR reaction 3: uses

oligonulceotide from 5' from PCR 1 and 3' from PCR 2. The product of the third PCR reaction was placed into TOPO (Invitrogen) then subcloned using NotI and Xho.

The middle of the construct was assembled in to pBluescript first: The central piece of HD was an initial Xba digest which cuts at two sites bp9103 and bp3782 followed by a Xho (bp655) and KpnI (bp 3782) digest leading to a subcloning in pBluescript (Invitrogen).

The 3' end: The final piece of the construct was created by PCR with primers containing a NOT1 site on the three prime end and PCR over the endogenous Xba at bp 9103: 5' oligonulceotide GATAGGATCAGGAAAGGCTTTCC; 3' oligonulceotide TATAGGTACCGCGGCCGCAGGTGGTGACCTTGTGGAC. The full length constructs were cut from pBluescript and cloned into pCDNA3.1 (Invitrogen), a mammalian expression vector using the NOT1 sites created at both ends of full length HD.

Cell Transfection and Determination of Cell Viability

Equal numbers of wild-type ST*Hdh*$^{Q7/Q7}$ cells were plated onto glass coverslips approximately 10 hours prior to transfection using lipofectAMINE^{TM} 2000 (Invitrogen, Carlsbad, CA). The pEGFP-N1 expression vector (Invitrogen, Carlsbad, CA) was co-transfected with p*HTT*[128Q] or the control vector at a 1:3 ratio to allow for estimates of transfection efficiencies. Approximately 70% of cells were GFP positive. Cells were treated with $MnCl_2$ 24 hours post-transfection and analyzed 30 hours after exposure. Coverslips were harvested, washed in PBT, fixed with 4% paraformaldehyde, washed

again, and mounted onto slides with ProLong Gold antifade reagent with DAPI nuclear stain (Invitrogen, Carlsbad, CA). DAPI positive cells were imaged by fluorescence microscopy using a Zeiss Axioplan microscope (Thornwood, NY) with a 10x objective by systematic unbiased sampling of non-overlapping fields. Number of surviving cells per field was quantified using NIH ImageJ by the threshold and analyze particles commands as previously described (Bowman et al. 2007).

Cell Survival Assays

ST*Hdh*$^{Q111/Q111}$ and wild-type ST*Hdh*$^{Q7/Q7}$ cells were plated at equal density (350000 cells per 10 cm^2) the evening before treatment. Cells were exposed to metals in the culture media for 26 to 30 hours. Cell viability was assessed with a hemacytometer using trypan blue exclusion or MTT assay. Trypan blue exclusion: Cells were harvested after exposure with trypsin and counted on a hemacytometer utilizing trypan blue exclusion. Survival data is graphed as the number of viable cells relative to the untreated for each genotype is shown as percent untreated. MTT assay: After treatment, the media was exchanged for 500 µl of 0.5% MTT salt in MEM (Invitrogen, Carlsbad, CA) containing FBS and Penicillin-Streptomycin for 4 hours. Then the media was removed and Sorenson's Buffer was diluted 2.5:20 in DMSO. 200 µl of Sorenson's Buffer in DMSO was added to the empty wells and the plates were returned to the incubator until all the MTT salt precipitate could no longer be visualized under a microscope. 60 µl from each well was removed and placed in a 96-well plate. Absorbance at 590 nm was read on a Tecan (Durham, NC) plate reader (Ehrich et al. 2000). The average absorbance relative to the untreated control is graphed as percent untreated.

Primary Culture from YAC128Q and Control Animals.

Briefly, postnatal day 0-1 pups were obtained from a cross of wild-type FVB and positive FVB-Tg(YAC128)53Hay/J (YAC128) animals, created by Michael Hayden and colleagues (University of British Columbia, Canada), and obtained from JAX (Bar Harbor, ME) (Slow et al. 2003; Tang et al. 2005). Cerebellum was removed and placed in ice cold Hank's Balanced Salt Solution (Invitrogen). Tissue was diced, passed through a series of reduced bore pasture pipettes, and digested with 0.25% trypsin (Sigma, St. Louis, MO) and 1 mg/ml Dnase I (Sigma, St. Louis, MO). After dissociation, enzymes were inhibited with warm Neurobasal media (Gibco, Carlsbad, Ca) containing 10% FBS (Sigma, St. Louis, MO). Cells were counted and plated at ~$1x10^6$ on to ether poly-D-lysine (Sigma, St. Louis, MO) coated or uncoated dishes. Neuronal media for cerebellar cultures contained DMEM (Mediatech), 15% FBS (Sigma, St. Louis, MO), B27 supplement (Gibco, Carlsbad, Ca), potassium chloride (Sigma, St. Louis, MO), L-glutamine (Sigma, St. Louis, MO), penicillin-streptomycin (Mediatech), glucose (Sigma, St. Louis, MO), pyruvate (Sigma, St. Louis, MO), and nonessential amino acids (Sigma, St. Louis, MO). The cells were then counted before being plated equally in replicate. Some cultures were placed onto poly-D-lysine treated dishes to enrich for neuronal attachment while others were placed onto uncoated plates to encourage the growth of astrocytes. Each dissection was maintained as a separate culture. Astrocytes from the striatum were initially cultured in Neurobasal medium (Invitrogen), 10% FBS, B27 supplement, 2mM glutamine, penicillin-streptomycin. After 12 days *in vitro*, neuronal cultures were exposed to Mn for 24 hours and cell death assayed by MTT method as described above. The astrocyte cultures were split at day 7 of culture and at this time all

cultures from each of each genotype were combined creating one culture from YAC128 and a one from wild-type control animals. The day after replating the astrocyte cultures the media was changed: Minimal Essential Media + earl's salts (Mediatech), 10% FBS, l glutamine, penicillin-streptomycin. Treatment protocol and assays were done as stated previously in the neuronal cultures.

Western Blots

Wild-type and mutant ST*Hdh* cell lines were plated evenly and treated with Mn as stated above, washed, and counted. The cell pellet was lysed in a volume of RIPA buffer with protease and phosphatase inhibitors proportional to the number of cells, and an equal volume of each sample was loaded onto duplicate polyacrylamide gels, one 10% and another 6%. Two gels were necessary due to the size differenced of the proteins. HTT levels were examined on the 6%; Actin, TfR, and Akt on the 10% gel. Western blots were visualized with Thermo Scientific Pierce Supersignal West Dura Extended Duration Chemiluminescent Substrate (Waltham, MA) on an Ultralum Omega 12iC (Claremont, CA). Quantification was done using ImageJ (NIH).

Colorimetric LDH Assays

We used the In Vitro Toxicology Assay Kit (TOX7) (Sigma, St. Louis, MO). ST*Hdh* cells were plated at equal density (350,000 per dish), exposed to the metal as indicated for 30 hours, and then lysed with Triton tx-100 according to the manufactor's protocol. Intracellular enzyme activity was measured at an absorbance of 490nm.

Statistical Analysis

Univariate and multivariate ANOVAs were performed using SPSS software (SPSS, Inc., Chicago, IL). All *post-hoc* analysis and pair-wise comparison's were done by Student's t-tests (two-tailed, assuming equal variances), error bars are expressed as standard error of the mean (SEM), all using Microsoft Excel (Redmond, WA).

Results

Phenotypic differences of ST*Hdh* Lines, Growth Rates and LDH Activity, Do Not Alter Mn Toxicity

The wild-type and mutant striatal cell lines are independent lines. Thus, differences in their response to Mn(II) exposure may be due to expression of mutant *HTT* or other inherent differences between the two lines. Because the mutant ST*Hdh* cells are known to divide at a slower rate than the wild-type ST$Hdh^{Q7/Q7}$ cells, we wanted to examine the role of cell division on Mn toxicity (Figure II.1.) (Trettel et al. 2000). A unique feature of the ST*Hdh* lines is that the SV40 large T antigen, which drives cell division, is temperature sensitive. Normal cell growth occurs at 33ºC while at a higher temperature of 39ºC cell division is blocked (Cattaneo et al. 1998). Cells were split. Some of the cultures were maintained at 33ºC and allowed to divide as normal. Other cells from the split were moved to the non-permissive temperature of 39º for 24 hours, and then both groups were exposed to Mn. Both mutant and wild-type cells show toxicity under standard culture conditions (33ºC) with Mn being less toxic in the mutant cell line (Figures II.4-6.). In cultures at 39ºC, where cell division is greatly reduced, both

cell lines displayed an increase in cell death in the absence of Mn (Figure III.1.A.). However, the wild-type cells continued to show more death in the presence of Mn when compared to the ST*Hdh*$^{Q111/Q111}$ line (Figure III.1.B.). Together, this data suggests that the variation in cell growth rates does not cause the *HTT*-Mn interaction.

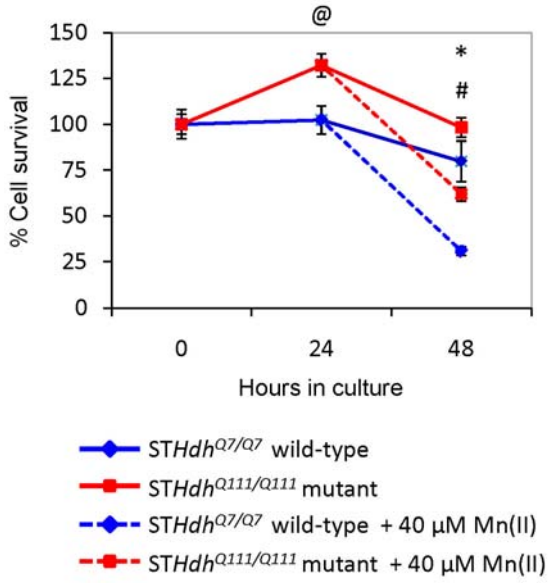


Figure III.1. Absence of Cell Growth Does Not Alter the Mn Phenotype. ST*Hdh* cells were plated at equal density, place at 39 ^{o}C for 24 hours to stop cell division and survival analyzed at indicated time or exposed to indicated amounts of Mn n=17 independent wells (±SEM). Significant differences within the wild-type unexposed samples when compared to 0 hour control (# p=0.05 t-test assuming unequal variance). Significant differences within the mutant unexposed samples when compared to 0 hour (@ p=0.05 t-test assuming unequal variance). Significant differences between the genotypes after Mn exposure (* p=0.05 t-test assuming unequal variance).

LDH activity is a common marker of cell viability. However, in the ST*Hdh* cells, microarray analysis has shown differences LDH RNA levels in the mutant line when compared to wild-type (Lee et al. 2007). We assayed the activity levels of this enzyme to explore any differences between the STHdh cell lines. To explore the ability of Mn to

alter LDH activity, we treated equal numbers of wild-type or mutant cells with indicated amounts of Mn(II) and examined LDH activity in the cell lysates. This pilot study indicates that at 40 µM Mn LDH activity increase specifically in the mutant cells compared to untreated ST*Hdh*$^{Q111/Q111}$. At 100 µM Mn, mutant Mn exposed cells have decreased LDH activity as do wild-type under all exposures concentrations (Figure III.2.). Student's t-test indicates significance from basal or unexposed cells within each genotype. However, the basal difference in the LDH activities between the two cell lines prompted us to examine LDH levels more closely. Equal numbers of mutant and wild-type cells show a significant reduction in the activity of LDH in the mutant cells which has been reported in other models of HD (Figure II.2.) (Fox et al. 2007). Due to these basal differences, we did not investigate the effect of Mn on LDH activity further.

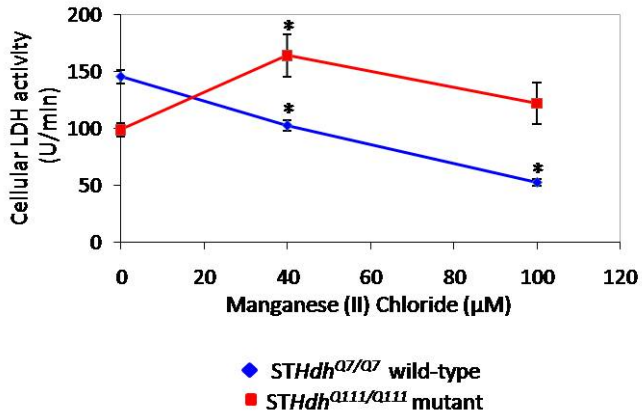


Figure III.2. Intracellular LDH Activity in ST*Hdh* Cells. Equal numbers of cells were plated, exposed to indicated amounts of Mn for 30 hours, and then internal LDH enzyme activity was measured.. N=2 independent wells (±SEM) . Significant differences in active when compared to basal levels within genotype (* p=0.05 t-test).

Expression of Mutant *HTT* is Sufficient for the Mn-Resistance Phenotype

To determine if the Mn(II) resistant phenotype is due to expression of mutant *HTT* we transiently expressed full-length human mutant *HTT* with 128 repeats (*HTT[128Q]*) in the wild-type ST*Hdh*$^{Q7/Q7}$cells. Transfected cells were examined for expression of mutant *HTT[128Q]* or wild-type *HTT[11Q]* constructs (Figure III.3.A.). Twenty-four hours after transfection with the control or *pHTT[128Q]* expression vector, cells were exposed to vehicle, 40 μM Mn(II), or 100 μM Mn(II) and cell survival assessed by microscopy. In addition to the *pHTT[128Q]* or control vector, cells were co-transfected with pEGFP-N1 at a 1:3 ratio to allow estimates of transfection efficiencies, approximately 70% of cells were GFP positive (Figure III.3.B.). Statistical analysis by two-way univariate ANOVA revealed a significant difference between *HTT[128Q]* and control transfected cells ($F_{(1,108)}$=7.33, p=0.008) and a significant two-way interaction between Mn(II) exposure and transfection ($F_{(2,108)}$=3.92, p=0.023). *Posthoc* analysis indicated significantly higher cell survival following Mn exposure in the *HTT[128Q]* transfected cells relative to control cells at both 40 μM (p=0.05) and 100 μM (p<0.001) Mn(II) exposures (Figure III.3C.). Therefore, expression of mutant *HTT* is sufficient to confer resistance to Mn(II) toxicity.

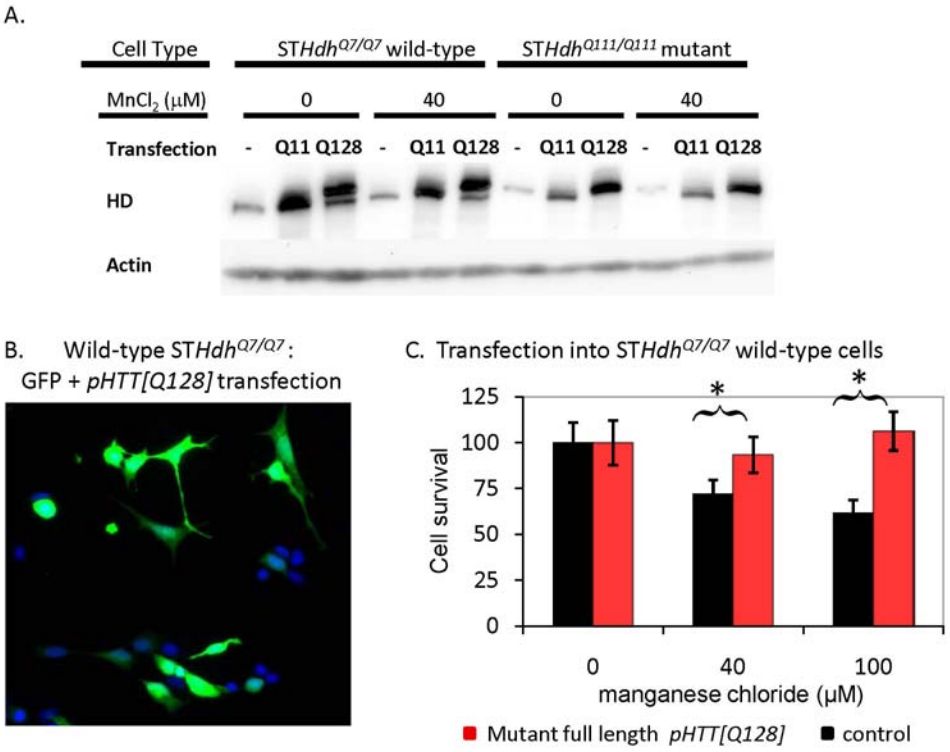


Figure III.3. Expression of Mutant *HTT* Confers Mn-Resistance Phenotype. A. Lysates harvested from transfection of full length *pHTT[11Q]* or *pHTT[128Q]* into wild-type ST*Hdh*$^{Q7/Q7}$ or mutant ST*Hdh*$^{Q111/Q111}$ cells. B. Cell images used to quantitate transfection efficiencies, approximately 70%. C. Graph of cell survival. Following exposure to Mn(II) chloride for 30 hours. The number of surviving cells per visual field (18 images per transfection/exposure group, 9 images from 2 independent coverslips) was counted by automated image analysis using NIH ImageJ software. Mean values are plotted as the percentage of cells relative to the average number of cells (± SEM) in vehicle exposed controls for each transfection condition, n=18 images. Significant differences in survival (* $p<0.05$ *post-hoc* t-test) between p*HTT[128Q]* expressing and control cells are indicated.

In the mutant cell lines, we saw no change in the resistance to Mn toxicity with the additional expression of the *HTT[128Q]* construct indicating that the lower levels of HD expression in the ST*Hdh*$^{Q111/Q111}$ cell line is not responsible for the lack of Mn sensitivity (Figure III.4. compare untransfected and *pHTT[128]*). The wild-type *HTT[11Q]* transfection into the mutant line suggests that the wild-type HD construct is

able to restore Mn sensitivity to the ST*Hdh*$^{Q111/Q111}$ cells (Figure III.4. compare control and *pHTT[Q11]* transfected); these preliminary results suggest that wild-type and mutant HD exert opposing effects on Mn sensitivity.

To examine the role of the polyglutamine domain in the phenotype, we transfected *HTT[128Q]* truncated expression vector into the wild-type cells. *HTT[128Q]* truncated, was also to increase the resistance of these cells to Mn toxicity, showing that the first third of the HTT protein is sufficient for the Mn interaction phenotype (Figure III.5.)

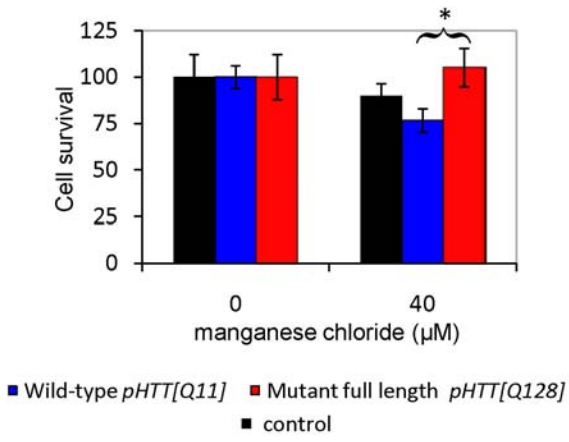


Figure III.4. Restoration of Mutant HTT Protein Levels Does Not Alter Mn Resistance in Mutant Cells. Transfection of p*HTT[128Q]* or p*HTT[Q11]* into ST*Hdh*$^{Q111/Q111}$cell line 24 hours before treatment with $MnCl_2$. Graphed as the percentage of cells remaining relative to the average number of survival without Mn exposure for each transfection condition, n= 18 (± SEM). Significant effect of genotype/transfection, exposure, or significant two-way interaction between genotype/transfection and exposure is designated by red numbering. * Significant difference in survival ($p<0.05$ *post-hoc t*-test assuming unequal variance) between control and *HTT[128Q]* transfected.

To explore further, the Mn-resistance phenotype of neurons expressing mutant *HTT*, we isolated primary neuronal cultures from the YAC128 mouse model of HD (Slow

et al. 2003). The YAC128 mice express full-length human *HTT* with 128 CAG repeats under control of the human *HTT* promoter on a yeast artificial chromosome. Both wild-type copies of the endogenous mouse *HTT* gene are present in the mutant animals (Slow et al. 2003). Primary cerebellar neuronal cultures from mutant YAC128Q animals have decreased sensitivity to Mn toxicity relative to cultures from wild-type animals (Figure III.6.). Therefore, mutant *HTT* protects against Mn toxicity in both striatal cell lines and primary neuronal culture models.

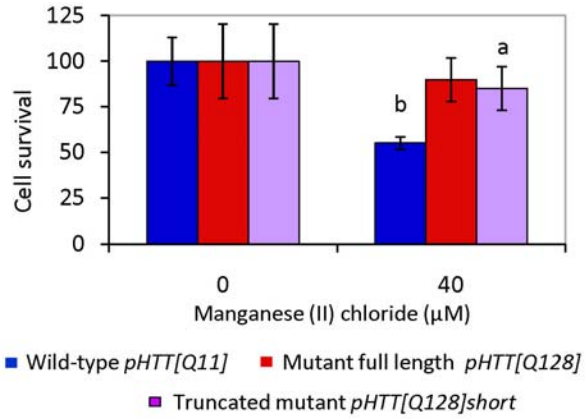
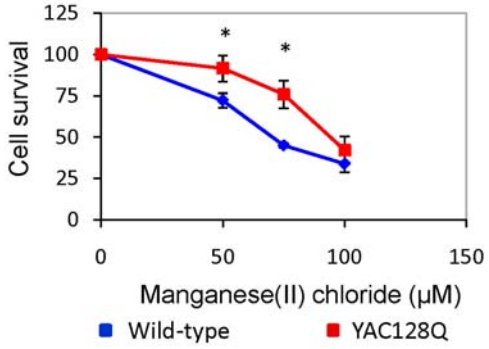


Figure III.5. Truncated HTT with Polyglutamine Domain is Sufficient to Confer Resistance. Transfection of p*HTT[128Q]* or p*HTT[128Q]short* into ST*Hdh*$^{Q7/Q7}$cell line 24 hours before treatment with $MnCl_2$. After exposure, coverslips stained with DAPI underwent systematic unbiased sampling of microscopy fields as previously described[63]. Graphed as the percentage of cells remaining relative to the average number of survival without Mn exposure for each transfection condition, n= 6 (± SEM). a compared to b Significant difference in survival ($p<0.01$ *post-hoc t*-test assuming unequal variance) between *HTT[11Q]* and *HTT[128Q]short* transfected.



ANOVA	F	Sig.
genotype	$F_{(1,48)}$=17.15	<0.001
Mn	$F_{(3,48)}$=57.71	<0.001
Mn* genotype	$F_{(3,48)}$=3.64	0.021

Figure III.6. Mutant *HTT* is Sufficient for Reduced Mn Sensitivity in Primary Cerebellar Cultures. Dissections were done from the brains of FVB wild-type or HD-YAC128Q litter-mates postnatal 1 animal. After 12 days, *in vitro* cells were treated with various concentrations of Mn(II) chloride for 48 hours. Cell survival was analyzed by MTT assay. n=6 independent wells, representative of 3 experiments. The average absorbance relative to the untreated control is graphed as percent untreated (± SEM). Tables show statistical analysis from a two-way univariate ANOVA. Significant effects of genotype/transfection, exposure, or two-way interaction of genotype/transfection by exposure are designated by red numbering. * Significant difference in survival ($p<0.05$ *post-hoc t*-test assuming unequal variance) between wild-type and YAC128Q cells.

Primary Cultures from YAC128 May Have a Cell Subtype Specific Responses to Mn Exposure.

Different cell types accumulated different amounts of Mn and astrocytes are known to accumulate approximately 80% of the total Mn within the brain (Wedler et al. 1984; Erikson et al. 2006; Morello et al. 2008). The ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q111/Q111}$ cell lines have been reported to be enriched in neuronal cell types (Trettel et al. 2000). To explore the ability of different cell types to affect the insensitivity of the HD mutant to Mn, we used primary cultures from the YAC128 mouse model of HD. Primary cultures that have been enriched for neuronal types by plating onto coated coverslips clearly display a differential sensitivity to Mn toxicity (Figure III.7.). Cultures plated onto uncoated dishes that enrich for astrocytes, do not show a decrease in cell death after Mn

exposure (Figure III.7.). The different responses of these broad subclasses of cells add strength to the argument that specific cell types are more vulnerable to Mn toxicity. However, cell staining is necessary to identify the cell types present in these cultures.

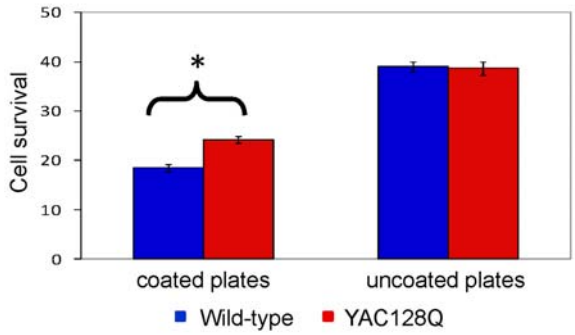


Figure III.7. A Subset of Brain Derived Cells from Mutant YAC128 HD Mice are Resistant to Mn Toxicity. Dissections were done from the cerebellum of FVB wild-type or HD-YAC128Q litter-mates postnatal 1 animal. Neuronal mixed cultures were plated onto coated coverslips and maintained for 12 days. Astrocytes were plated onto uncoated dishes and split on day 7. *In vitro* both cultures were treated with 100μM Manganese chloride 24 hours. The number of cells surviving was analyzed by MTT assay. n=6 independent cultures. The average absorbance relative to the untreated control is graphed as percent untreated (± SEM). * Significant difference in survival ($p<0.0001$ *post-hoc t*-test assuming unequal variance) between wild-type and YAC128Q cells.

40 μM Mn(II) Exposure Does Not Significantly Alter HTT Protein Levels

The *HTT* gene has been shown to be Fe-responsive and Mn(II) exposure is known to alter cellular Fe levels (Zheng et al. 1999; Hilditch-Maguire et al. 2000). Given these findings and HTT's ability to influence cell survival, we examined HTT to determine if Mn can alter protein levels, which may alter cell viability (Figure III.8.). Quantitative analysis demonstrated that HTT levels were not significantly altered in wild-type or mutant striatal cells after exposure to 40 μM Mn(II) for 30 hours. As previously

reported, mutant HTT protein levels are decreased in the ST*Hdh*$^{Q111/Q111}$ cells relative to wild-type protein levels in the wild-type cell line (Figure III.3.) (Trettel et al. 2000).

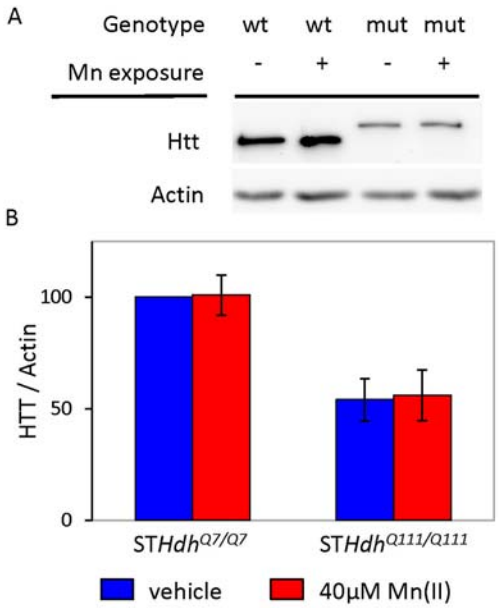


Figure III.8. Mn(II) Exposure Does Not Alter HTT Protein Levels in Striatal Cells. (A) representative blot showing lysates from ST*Hdh*$^{Q7/Q7}$ (wild-type) or ST*Hdh*$^{Q111/Q111}$ (mutant) cells after 30 hours of 40µM Mn(II) chloride exposure (+). (B) Quantification of HTT protein expression in striatal cell lines relative to actin. Mean values are plotted as a percentage of the vehicle exposed wild-type cells (± SEM. n=7 independent samples). Statistical analysis of HTT protein levels by two-way univariate ANOVA found a significant effect of genotype ($F_{(1,28)}$=28.1, $p<0.001$), but indicated no significant effect of Mn(II) exposure ($F_{(1,28)}$=1.13, $p=0.298$) or a genotype by exposure interaction ($F_{(1,28)}$=1.16, $p=0.292$).

Diminished Akt Activation in Response to Mn(II) Exposure in HD Striatal Cells

As changes in S473-phosphorylated Akt (pAkt) levels are associated with both Mn toxicity and HD, we hypothesized that this signaling pathway would show disease-toxicant interactions (Humbert et al. 2002; Gines et al. 2003; Colin et al. 2005; Warby et al. 2005). To begin we quantified pAkt levels following a 30 hour exposure to 40 µM Mn(II). This exposure led to a Mn-dependent increase in pAkt levels ($p<0.05$) in wild-type cells, yet mutant cells showed no detectable change in pAkt levels relative to

untreated mutant cells (Figure III.9.). As expected from previous work, pAkt levels were elevated in the mutant line versus wild-type in the absence of Mn (Gines et al. 2003). Given this, it is possible that the failure to detect a Mn-dependent increase in pAkt was due to a general inability of these cells to increase pAkt levels beyond this point.

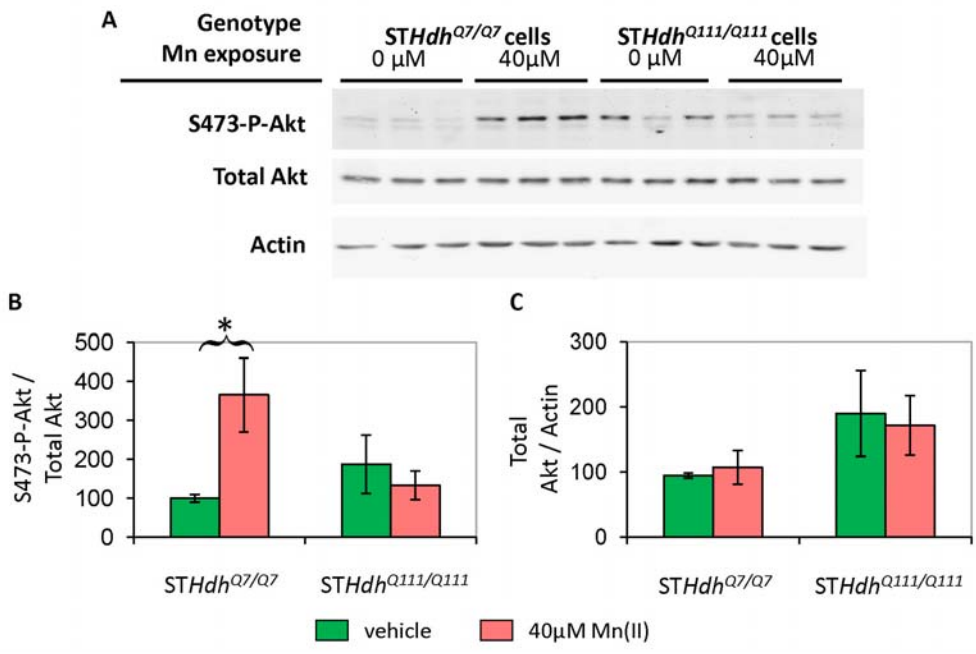


Figure III.9. Diminished Mn-Dependent AKT Phosphorylation in HD Striatal Cells. Lysates harvested from ST*Hdh*$^{Q7/Q7}$ (wild-type) or ST*Hdh*$^{Q111/Q111}$ (mutant) cell lines after 30 hours of manganese chloride exposure were analyzed by western blot for total Akt (Akt), phosphorylated Akt (S473-P-Akt), and actin. (A) Representative blot showing 3 samples of each genotype-exposure group. (B-C) Quantification of protein expression in striatal cell lines, with (B) S473-P-Akt normalized to total Akt, and (C) total Akt normalized to actin. Mean values are plotted as a percentage relative to the vehicle exposed wild-type cells (± SEM, n=7 independent samples). Significant differences in protein levels (* $p<0.05$ *posthoc t*-test) between vehicle and Mn-exposed cells are indicated

To determine if higher levels of Mn are capable of increasing pAkt levels, we exposed cells for a shorter time point (3 hours) to allow us to collect protein before significant cell death. At this time point, other studies reported strong Mn-dependent

increases in pAkt levels (Bae et al. 2006; Lee et al. 2009). Mutant ST*Hdh*$^{Q111/Q111}$ cells were capable of increasing pAkt levels in response to high concentrations of Mn(II) (Figure III.11.). However, in the wild-type cells 40 μM Mn(II) was sufficient to significantly increase pAkt above basal levels ($p>0.05$) (Figure III.10.A. and III.11.). We did not detect a significant increase in pAkt above its higher basal levels in mutant cells until 200 μM Mn(II) or higher ($p>0.05$) (Figure III.10.A. and III.11.). Interestingly, at exposures of 100 μM and above, the levels of pAkt in the wild-type cells increased to equal the levels seen in mutant cells exposed at the same Mn concentration (Figure III.10.C.). Controlling for the elevated pAkt levels in the mutant by normalizing the basal levels of each genotype to 100%, mutant cells exhibited a significant decrease in pAkt activation relative to wild-type cells across all tested Mn concentrations ($p>0.05$) (Figure III.10.B.).

Enhanced Akt Activation in Response to Cd(II) Exposure in HD Striatal Cells

To evaluate whether the reduced phosphorylation of Akt by Mn(II) exposure in mutant cells is acting as a specific marker of the Mn-HD interaction, we tested the effect of Cd(II) exposure on pAkt levels in the ST*Hdh* cell lines. We tested Cd given its opposite effect from Mn on cell survival (Figure II.4.). Published evidence has demonstrated that Cd(II) exposure leads to phosphorylation of Akt at S473 in a variety of cell types (Thevenod 2009). A 3 hour exposure of ST*Hdh* cells to 50 μM Cd(II) increased pAkt levels in both wild-type and mutant lines (Figure III.10.A. and B.). Indeed, despite the elevated basal levels of pAkt, Cd(II) exposed mutant ST*Hdh*$^{Q111/Q111}$ cells had significantly higher levels of pAkt than wild-type cells ($p<0.05$) (Figure III.11.).

Controlling for the difference in basal pAkt levels, mutant and wild-type cells both had a similar ~2-fold increase in pAkt levels (Figure III.10.B.). Thus the elevated basal pAkt levels in the mutant cells does not limit *per se* the capacity of mutant cells to increase the pAkt to total Akt ratio.

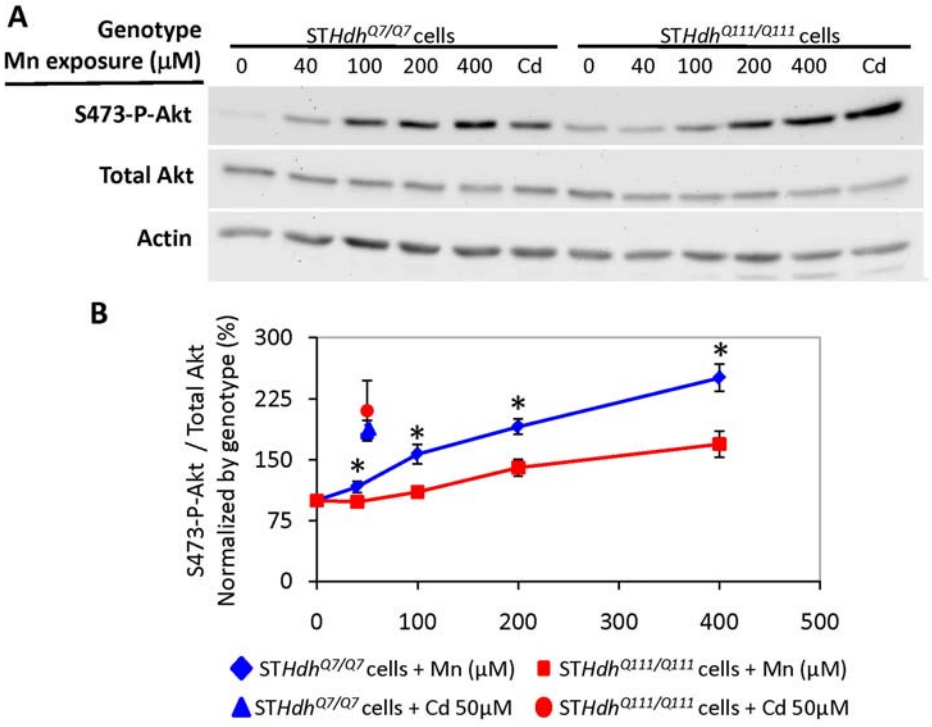


Figure III.10. Diminished Mn-Dependent Akt Phosphorylation in HD Striatal Cells. (A) Lysates harvested from ST*Hdh*$^{Q7/Q7}$ (wild-type) or ST*Hdh*$^{Q111/Q111}$ (mutant) cells after 3 hours of manganese chloride exposure were analyzed by western blot for phosphorylated Akt (S473-P-Akt), total Akt, and actin. Representative blots are shown. (B) Quantification of S473-P-Akt /total Akt expression in striatal cell lines. Mean values were normalized by genotype to the vehicle-only control**.** (± SEM, n=4 independent samples). Significant differences in protein levels (* $p<0.05$ *posthoc t*-test) between genotypes are indicated for each exposure level.

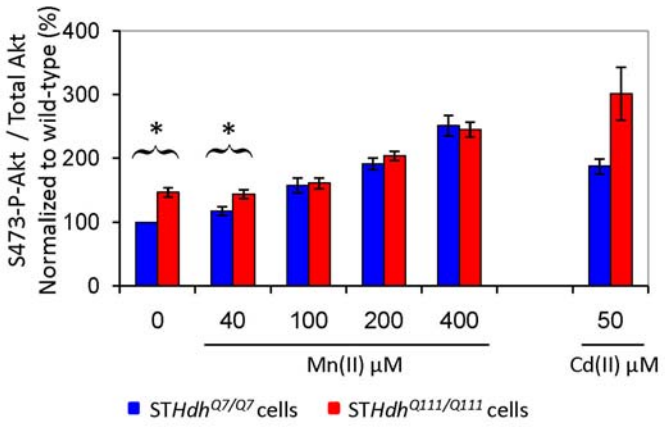


Figure III.11. Diminished Mn-Dependent Akt Phosphorylation in HD Striatal Cells. Same data as III.10 regraphed. Mean S473-P-Akt /total Akt expression ratio values were normalized to the wild-type vehicle control (± SEM, n=4 independent samples). Significant differences in protein levels (* $p<0.05$ *posthoc t*-test) between genotypes are indicated for each exposure level. In collaboration with Michal Wegrzynowicz

Conclusions

The resistance to Mn toxicity can be conferred to cells expressing wild-type HTT by overexpression of the mutant protein and indicates that the different responses to Mn between the wild-type and mutant striatal cell lines is not due to some inherent difference of the two lines, such as cell division rates and LDH activity (Figures II.1. and 2.). Additionally, the gene-environment interaction still exists in the absence of cell division (Figure III.1.). Overexpression of the full-length HD construct increases protein levels above the endogenous and validates that the differential response is due to expression the mutant protein and not because of the low expression of mutant protein in the ST*Hdh*$^{Q111/Q111}$ cells (Figures III.3.A. and III.8.). Perhaps even more telling is that over-expression of a wild-type HD construct in the mutant cells can confer sensitivity to Mn in cells that normally are resistant, implicating a loss of function in the mutant cells which can be overcome by over-expression of the wild-type protein (Figure III.4.). Our

preliminary domain mapping studies have shown that the first third of HTT, which contains the mutation, is sufficient for the resistance to Mn (Figure III.5.). Additionally, primary culture data also has shown that the polyglutamine expansion reduces Mn toxicity in another cellular model of HD (Figure III.6.). Our data also indicates that neuronal populations, not astrocytes are more sensitive to Mn (Figure III.7.). The absence of differential toxicity in the astrocytic culture may reflect the tendency of these cells to accumulate more Mn. Astrocytes utilize Mn differently than neurons and this may play a role in the different survivals we have reported. For example, astrocytes accumulate Mn in the enzyme, GS that is not expressed in neurons (Wedler et al. 1984). Finally, we report a blunted response to the Akt cell-signaling pathway (Figures III.9., 10, and 11.).

Following up on the discovery that expression of the disease-causing allele of *HTT* is protective against Mn toxicity, we found no evidence that HTT protein levels are altered by 40μM Mn(II) exposure, indicating that survival differences between cells does not depend on a reduction of the toxic mutant HTT protein levels or an increase of wild-type expression (Figure III.8.). However, higher levels of Mn(II) exposure may be able to alter the expression of *HTT*. Importantly, dosimetry studies have revealed that Mn concentrations in rodent striatum are normally between 4 μM to 18 μM, and can increase to as high as 70 μM in Mn(II) exposed animals (Aschner et al. 2005). Thus, our observation of a *HTT*-Mn gene-environment interaction at Mn(II) exposures in this range is potentially pathologically relevant.

LDH activity data indicates that low levels of Mn may increase the activity of the enzyme in the ST*Hdh*$^{Q111/Q111}$ cells. Changes in both LDH protein level and activity have been reported in multiple HD model including the ST*Hdh*$^{Q111/Q111}$ cell line (Fox et al. 2007; Lee et al. 2007). LDH is part of the cellular energy metabolism pathway, converting pyruvate to lactate (Kasischke et al. 2004). Mn is known to increase the activity of this enzyme in immortalized cell lines (Reaney et al. 2005). The relationship between low LDH level observed in HD models and Mn ability to improve enzyme function must be studied more thoroughly. Given that the ST*Hdh*$^{Q111/Q111}$ cells have been reported to have a reduced levels of energetic substrates which reduce the function of mitochondria, this may provide a mechanism for improvement in survival we reported with low level exposures of Mn (Figure II.2, 7, and III.2,) (Milakovic et al. 2005). In addition, Mn has been reported to stimulate the electron transport chain at low level exposures, which could improve survival in the energy deficient ST*Hdh*$^{Q111/Q111}$ cells.

Cadmium and 3NPA exert their toxic effects directly on the mitochondria (Li et al. 2003; Mao et al. 2006). The mutant ST*Hdh*$^{Q111/Q111}$ cells are reported to have an increased susceptibility to multiple mitochondrial stressors (Gines et al. 2003; Seong et al. 2005; Oliveira et al. 2006). More recently, Cd(II) toxicity has been linked to aberrant activation of mitogen-activated protein kinases (MAPK) and the mammalian target of rapamycin (mTOR) cell signaling pathways (Lopez et al. 2006; Chen et al. 2008). Disruption of these signaling pathways has also been suggested in HD (Ravikumar et al. 2004; Apostol et al. 2006). Further investigation is needed to determine the relative contribution of these and other mechanisms to the increased sensitivity of ST*Hdh*$^{Q111/Q111}$ cells to Cd(II) toxicity.

Activation of Akt via S-473 phosphorylation has been associated with neuroprotection (Brunet et al. 2001). Yet, we found that the increased sensitivity of mutant ST*Hdh*$^{Q111/Q111}$ cells to Cd(II) toxicity correlated with increased S473-P-Akt levels relative to wild-type exposed cells, while the decreased sensitivity to Mn(II) toxicity correlated with a blunted S473-P-Akt response (Figure III.10.). While this observation does not rule out a neuroprotective role for Akt signaling in Mn(II) or Cd(II) toxicity, it does demonstrate that enhanced Akt activation between wild-type and HD mutant cells does not correlate with improved cell survival following exposure to these environmental toxicants in the ST*Hdh* cellular model. Future studies are needed to explore changes in Akt signaling following exposure to these metals in animal models of HD. In addition to activation of Akt at S-473, this protein can dually be regulated by another phosphorylation site T-308 (Datta et al. 1999) . The maximal activity of Akt requires phosphorylation at both of these residues (Jacinto et al. 2006). However, these two residues have been shown to be differentially regulated. For example, T-308 phosphorylation is not altered by the drug rapamycin but rapamycin blocks S-473 phosphorylation (Malagelada et al.). The role of T-308 phosphorylation of Akt in HD had not been thoroughly studied and should be investigated in this model.

Besides Akt, several other kinases are reported to be activated upon Mn exposure including ERK (Extracellular signal-regulated kinase), p38, and JNK (c-Jun N-terminal kinase) (Bae et al. 2006; Moreno et al. 2008; Lee et al. 2009). Additionally, Cd has been shown to affect MAPK and ERK1/2 signaling in hippocampal slices (Rigon et al. 2008). It will be interesting to evaluate how HD influences activation of these and other signaling systems that are modulated by Mn and Cd.

CHAPTER IV

MUTANT HUNTNGTIN IMPAIRS MANGANESE ACCUMULATION TO LIMIT ITS TOXICITY

Introduction

The divalent metal Mn is essential for proper cellular function, as it is required for the activity of many enzymes. Mn-dependent enzymes are important to many diverse cellular functions, playing essential roles in amino acid, lipid, protein, and carbohydrate metabolism. The central nervous system relies on Mn as a cofactor for several key enzymes such as glutamine synthetase, pyruvate decarboxylase, superoxide dismutase 2, arginase, and serine/threonine protein phosphatase I (Christianson 1997; Takeda 2003). Though an essential nutrient, overexposure to Mn can lead to a neurodegenerative disorder called manganism.

Patients with Manganism present with dystonia and hypokinetic movement symptoms similar to idiopathic PD (Keen et al. 2000; Olanow 2004; Aschner et al. 2009). Even though Mn overexposure is a known risk factor for PD, individuals continue to be exposed in various industrial settings, for example in the welding, mining, steel production, and automotive repair industries (Cotzias 1958; Roth et al. 2003; Weiss 2006). The metal accumulates in the basal ganglia leading to neurodegeneration and gliosis of the substantia nigra, globus pallidus, and striatum (Pal et al. 1999; Erikson et al. 2003).

The primary cellular transporter of Mn is the divalent metal transporter (DMT1) but calcium and Fe transporters (e.g. TfR) move Mn as well. (Gunshin et al. 1997; Malecki et al. 1999; Garrick et al. 2003). In addition, the citrate transporter and ZIP-8, a metal/bicarbonate ion symporter, have been shown to transport Mn (Davidsson et al. 1989; Aschner et al. 1994; Crossgrove et al. 2003; He et al. 2006). However, the transporters move multiple metals in addition to Mn with varying affinities. The divalent metal transporter, DMT1 transports Fe, Zn, Cd, Co (cobalt) and Cu (Garrick et al. 2006). The Zip8 family members, Zip8 and Zip14, have both been shown to transport Cd (He et al. 2006; Girijashanker et al. 2008).

In chapter II, we reported a gene-environment interaction between the protein HTT and Mn, discovered through a metal toxicity screen. The toxicity of Fe(III), Cu(II), Pb(II), Co(II), Zn(II), and Ni(II) were reported to be unchanged in the presence of the mutation (Figure II.4.)(Williams et al. 2009). HTT is mutated in the autosomal dominant disorder HD. HD causes neurodegeneration of the brain including the basal ganglia where Mn accumulates. Additional studies of this interaction, revealed a limited response of the Akt cell signaling pathway to Mn in the presence of the HD mutation (Figure III.8-11.). Both Mn exposure and mutant HTT are known to alter the activity of Akt (Humbert et al. 2002; Gines et al. 2003; Colin et al. 2005; Warby et al. 2005). Mn fails to cause efficient phosphorylation of Akt S-473 in cell expressing mutant HTT (Figure III.8.-11.). Importantly, the Akt pathway was robustly activated in the presence of Cd, indicating that the Akt pathway can respond to stimulation in the presence of the mutation (Figure III.10. and 11.).

Because the Akt pathway shows a limited response to Mn in the mutant cell line, we propose that the accumulation of Mn within the ST*Hdh* cells may be altered. Metal accumulation within a cell can be altered by several mechanisms: (1) a reduction in transport or uptake, (2) an inability to store or accumulated metals within the cell, or (3) an increase in export. Currently, very little is known about the cellular export of Mn in eukaryotes and prokaryotic export pathways are not Mn exclusive, transporting multiple divalent metals (Rosch et al. 2009). Also, previously published data suggest that the efflux of Mn from the brain slices may be due to passive diffusion (Yokel et al. 2003). Given that, the effect of mutant *HTT* on Mn transport is highly selective to the metal Mn; our data indicate Mn specific transport mechanism behind the gene-environment interaction.

As mentioned above, both DMT1 and the TfR pathway are possible pathways of Mn entry into the cell. The purpose of TfR is to move Fe^{+3} through the process of endocytosis but it can also move Mn in its 3+ oxidation state (Aschner et al. 1994; Malecki et al. 1999; Malecki 2001). Fe^{+3} binds to the protein ferritin allowing a conformational change which allows binding to the cell surface receptor TfR. The complex is then is endocytosed, where the metal is released and the receptor recycled to the cell surface (Roth et al. 2003; Bressler et al. 2007). Additionally, *HTT* has been functionally linked to Fe homeostasis and mutations in a key Fe storage protein can cause an HD-like disorder (Trettel et al. 2000; Cozzi et al. 2006; Simmons et al. 2007). Several studies have also revealed elevated levels of Fe in the striatum of HD patients and animal models (Dexter et al. 1991; Fox et al. 2007). Our previous results led us to hypothesize

that the gene-environment interaction between *HTT* and the metal Mn results in a reduction in Mn levels and that differences are due to impairment of Mn transport.

Methods

Chemicals, Reagents, and Cell Culture Supplies

Unless indicated tissue culture media and supplements were obtained from Mediatech (Manassas, VA). The clonal striatal cell were a acquired from Marcy Macdonald, PhD (Massachusetts General Hospital, MA), cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), L-glutamine, 400 μg/ml G418 and Penicillin-Streptomycin. Mn(II) chloride and Ni(II) chloride were from Alfa Aesar (Ward Hill, MA) and Fe(III) chloride was from Sigma (St. Louis, MO). Buffers for MTT assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt (VWR, West Chester, PA), Sorenson's Buffer (0.1 M glycine, 0.1 M $NaCl_2$, pH 10.5), dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO).

Cell Survival Assays

The ST*Hdh* cells were cultured at 33ºC (Cattaneo et al. 1998; Trettel et al. 2000). For the assays, ST$Hdh^{Q111/Q111}$ and wild-type ST$Hdh^{Q7/Q7}$ cells were plated at equal density (350000/plate) the evening before treatment. The next morning, culture media with the addition of metals, and cells were exposed for 30 hours. Cell viability was

assessed by MTT assay following established protocols (Ehrich et al. 2000). After exposure as stated above, media was replaced with 500 µl of 0.5% MTT salt in Minimal Essential Medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with FBS and Penicillin-Streptomycin. Cell were cultured with MTT for four hours and then exchanged for 200 µl of Sorenson's Buffer diluted 2.5ml into 20 ml DMSO. The plates were returned to the incubator until all the MTT salt precipitate could no longer be visualized under a microscope. 60 µl from each well was removed and placed in a 96-well plate, and absorbance read at 590 nm. Cell survival data was normalized by genotype to the vehicle-only exposed control included in each independent sample set.

Antibodies

TfR 1:500 (Invitrogen, Carlsbad, CA), and actin 1:2000 (Developmental Studies Hybridoma Bank, Iowa City, IA). IgG mouse secondary antibody was from Jackson Immunoresearch Laboratories (West Grove, PA) and was used at 1:15000.

Westerns Blot

Equal numbers of wild-type and mutant ST*Hdh* cells were plated (350000/plate) and treated with Mn(II) 30 hours. The cell pellets were lysed in RIPA buffer (50mM Tris, 150mM $NaCl_2$, 0.1% SDS, 1.0% Nonidet 40, 12mM deoxcholic Acid, 1x protease inhibitor cocktail (Sigma), 1x phosphatase inhibitor cocktails I and II (Sigma) and loaded by equal cell number for SDS-PAGE. Western blots were visualized with Thermo

Scientific Pierce Supersignal West Dura Extended Duration Chemiluminescent Substrate (Waltham, MA) on an Ultralum Omega 12iC (Claremont, CA). Measurements of integrated density of protein bands were performed with ImageJ (NIH), with background correction calculated using a signal ratio error model, as described (Kreutz et al. 2007). Calculations of relative signal were normalized to untreated wild-type or mutant sample for each set, as indicated.

Graphite Furnace Atomic Absorption Spectroscopy (GFAAS)

Mn and Fe concentrations were measured with GFAAS (Varian Inc., AA240, Palo Alto, CA). ST*Hdh* cells were cultured and treated as described above for cell viability assays, harvested by trypsinization, washed multiple times in PBS and then flash-frozen until analysis. For analysis, cell pellets were thawed and digested in 200 µl ultrapure nitric acid for 24 hours in a sandbath (60°C). Mn content was determined by the following protocol: A 20 µl aliquot of the digested sample was brought to 1 ml total volume with 2% nitric acid for analysis. Bovine liver (NBS Standard Reference Material, USDC, Washington, DC) (10 µg Mn/g) was digested in ultrapure nitric acid and used as an internal standard for analysis (final concentration 10 µg Mn/L*)* as published previously (Anderson et al. 2009).

Animal Mn-Exposure

All animal studies strictly followed protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee and were adopted to minimize pain and distress of the animals. The FVB-Tg(YAC128)53Hay/J mouse line (YAC128Q)

was obtained from JAX (#004938, Bar Harbor, ME). The Mn exposure protocol followed a previously published paradigm (Dodd et al. 2005). In brief, 3-month old YAC128Q HD mutant mice and their wild-type littermates were injected subcutaneously with 50mg/Kg Mn chloride tetrahydrate. All injections were done blind to genotype. Injections were carried out on days 0, 3, and 7. On day 8, the animals were sacrificed by cervical dislocation, and the brain regions were dissected, tails clipped for genotyping, and trunk blood collected into heparin-coated tubes. The tissues were then flash-frozen until analysis of metal content. Analysis of metal content by GFAAS was performed blind to genotype and exposure. Genotyping was done according to a previously published method (from JAX, 004938, Bar Harbor, ME) (Slow et al. 2003).

Statistical Analysis

Univariate, multivariate, and repeated measures ANOVA were performed using SPSS software (SPSS, Inc., Chicago, IL). *Post-hoc* analysis and pair-wise comparisons were done using Microsoft Excel (Redmond, WA) by Student's t-tests (two-tailed), except for comparison of normalized data versus control which were performed by testing for non-overlap of the 95% confidence interval, error bars are expressed as standard error of the mean (SEM).

Results

Cellular Mn Accumulation is Substantially Impaired by Mutant *HTT*

Given the severely blunted S473-P-Akt response of the mutant cells to Mn(II) exposure, we tested the hypothesis that the expression of mutant *HTT* impedes the cellular stress response to Mn(II) by decreasing accumulation of Mn during exposure (Figure III.8-11). We measured total intracellular Mn and Fe levels following Mn(II) exposure in the ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q111/Q111}$ cell lines by GFAAS (Garcia et al. 2007). We observed that the ST*Hdh*$^{Q111/Q111}$ cells accumulated 4-fold ($p<0.005$) and 10-fold ($p<0.001$) less Mn after a 30 hour exposure to 40 μM Mn(II) and 100 μM Mn(II) respectively (Figure IV.1.A.). No difference in basal Mn levels was seen. However, the Mn levels in the vehicle-only samples were near the lower detection limit of the GFAAS. We retested basal levels of Mn in the STHdh cells by increasing the number of cells and therefore increasing levels of Mn above the detection limits of GFAAS. The ST*Hdh*$^{Q111/Q111}$ cells were found to accumulate significantly less Mn under standard cell culture conditions (Figure IV.2.). Under these conditions, Mn levels in the wild-type cells were 3.92 fmol per 100 cells (~0.189 nmol/mg protein), whereas in the mutant cells basal levels were lower at 1.12 fmol per 100 cells (~0.069 nmol/mg protein). These data strongly indicate that a deficit in net Mn uptake is, at least in part, the source of the resistance to Mn toxicity in the ST*Hdh*$^{Q111/Q111}$ cells.

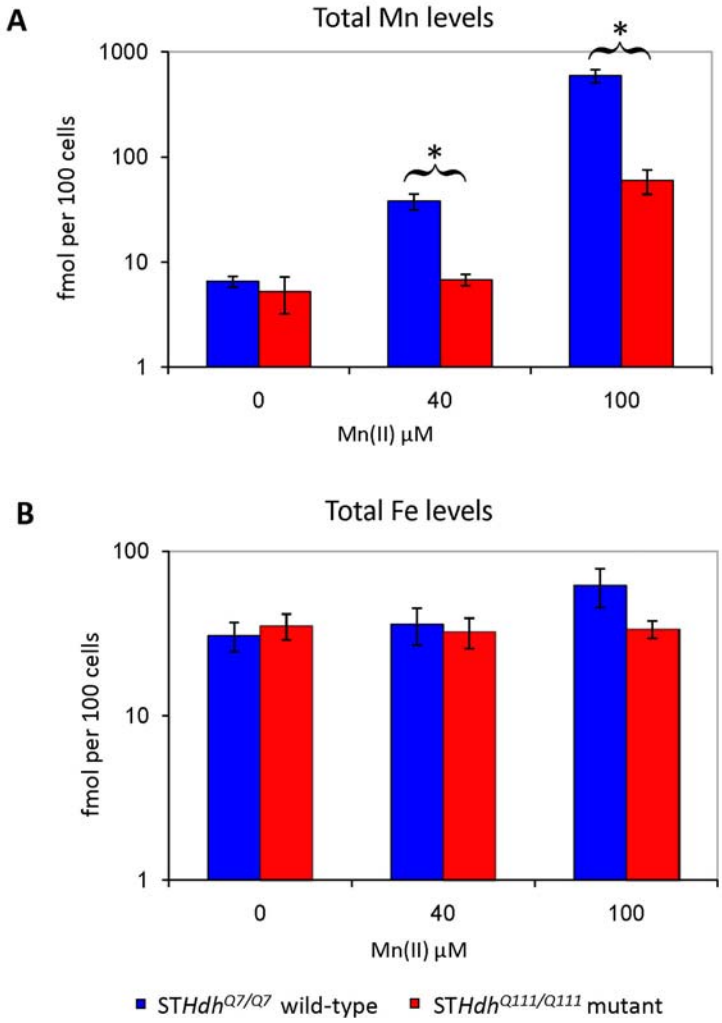


Figure IV.1. Substantial Decrease in Mn Accumulation in HD Striatal Cells. Measurement of total intracellular manganese in (blue) ST*Hdh*$^{Q7/Q7}$or (red) ST*Hdh*$^{Q111/Q111}$ cell lines. (A) The average amount of intracellular Mn is plotted on log scale (± SEM, n=5 independent samples). (B) Measurement of total intracellular Fe levels in same cells. The average amount of intracellular iron is plotted on log scale (± SEM, n=5 independent samples). Significant differences in metal levels (* $p<0.05$ *post-hoc* t-test) between wild-type and mutant ST*Hdh* cells for each exposure are indicated.

Striatal Specific Deficit in Total Mn Accumulation in YAC128Q HD Mouse Model

To determine if expression of mutant *HTT* influences Mn accumulation *in vivo*, we exposed 3-month (presymptomatic) YAC128Q HD mice and wild-type littermates to Mn(II) using a subcutaneous Mn exposure paradigm (Slow et al. 2003; Dodd et al. 2005).

Loss of striatal volume in the YAC128Q HD mouse model is absent as late as 6 months of age, and is first detected at 9 months of age (Slow et al. 2003). Analyses of cerebellar, cortical, hippocampal, and striatal Mn levels by GFAAS indicated that Mn(II) exposed wild-type animals had increased total Mn levels in all four brain regions with the striatum having the greatest accumulation (Figure IV.3.). Cerebellum, cortex, and hippocampus showed similar increases in Mn levels between wild-type and mutant animals, while striatum from wild-type Mn(II) exposed animals showed higher levels than mutant (1.51 mol Mn/mg protein versus 0.92 mol Mn/mg protein). A multivariate two-way ANOVA was used to analyze regional Mn levels (Table IV.1). ANOVA found a significant effect of Mn(II) exposure for each of the four brain regions ($p<0.05$). A significant effect of genotype and a genotype by exposure two-way interaction was found for striatum only ($p<0.05$). *Post-hoc* analysis of striatal Mn levels showed significantly less ($p<0.05$) Mn accumulation in mutant versus wild-type Mn(II) exposed animals (Figure IV.3.).

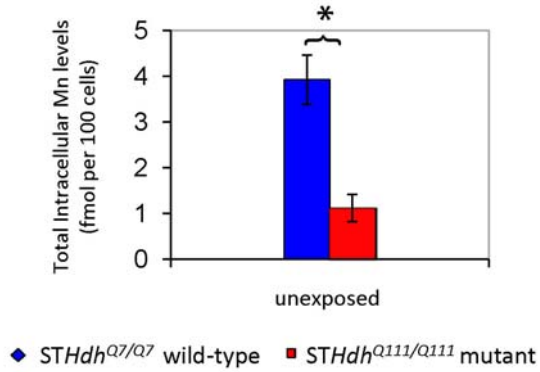


Figure IV.2. Low Basal Levels of Mn in Mutant ST*Hdh* Cells. Measurement of total intracellular Mn in (Blue) ST$Hdh^{Q7/Q7}$ or (Red) ST$Hdh^{Q111/Q111}$ cell lines after under standard cell culture conditions. The mean total metal levels (± std error) are plotted as indicated. * significant difference in survival ($p<0.05$ t-test assuming unequal variance) between wild-type and mutant cells.

We have confirmed the impaired striatal Mn accumulation in the YAC128Q HD animals in an independent set of animals by inductively coupled plasma mass spectrometry. Therefore, a mouse model of HD that recapitulates the selective degeneration of the corpus striatum observed in patients, also exhibited an early and selective deficit in striatal Mn accumulation after Mn(II) exposure. We also examined Fe levels in the same brains and analyzed by the same multivariate two-way ANOVA model (Figure IV.4. and Table IV.2.). The analysis failed to find a significant effect of genotype, Mn-exposure, or two-way genotype by Mn-exposure interaction for Fe levels in any brain region.

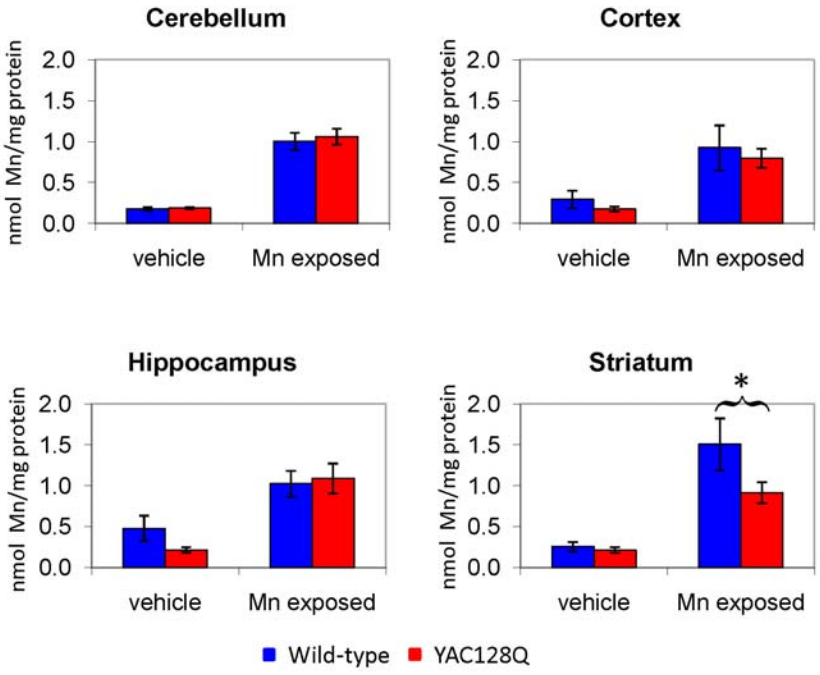


Figure IV.3. Reduced Striatal Mn Uptake in the YAC128Q HD Mouse Model. Wild-type and YAC128Q HD mice exposed to Mn(II). Mean manganese levels ± SEM are shown (n = 12 to 14 samples for each genotype-exposure group; 53 animals total). ANOVA analyses of total manganese levels see table IV.1. *Post-hoc* analysis of the genotype effect found a significant difference in striatal manganese accumulation between wild-type and mutant animals as indicated (* $p<0.05$ *post-hoc t*-test).

Factor	Brain Region	F	Significance
Genotype	Cortex	$F_{(1,53)}$=0.385	0.538
	Striatum	$F_{(1,53)}$=4.901	* 0.032
	Hippocampus	$F_{(1,53)}$=1.167	0.286
	Cerebellum	$F_{(1,53)}$=0.015	0.904
Mn	Cortex	$F_{(1,53)}$=15.921	* <0.001
	Striatum	$F_{(1,53)}$=10.387	* 0.002
	Hippocampus	$F_{(1,53)}$=6.057	* 0.018
	Cerebellum	$F_{(1,53)}$=72.399	* <0.001
Genotype x Mn	Cortex	$F_{(1,53)}$=0.063	0.803
	Striatum	$F_{(1,53)}$=4.504	* 0.039
	Hippocampus	$F_{(1,53)}$=0.385	0.538
	Cerebellum	$F_{(1,53)}$=0.099	0.754

Table IV.1. Analysis of Regional Mn Levels by Multivariate 2-Way ANOVA. ANOVA analysis of total manganese levels showed a significant effect of Mn(II) exposure for all four brain regions ($p<0.05$). A significant genotype and genotype by Mn(II) exposure two-way interaction was found for the striatum only ($p<0.05$). *Post-hoc* analysis of the exposure effect showed a significant increase in manganese levels in Mn(II)-exposed animals compared to vehicle-only animals of the same genotype ($p<0.05$ *post-hoc t*-test, not indicated). *Post-hoc* analysis of the genotype effect found a significant difference in striatal manganese accumulation between wild-type and mutant animals as indicated (* $p<0.05$ *post-hoc t*-test).

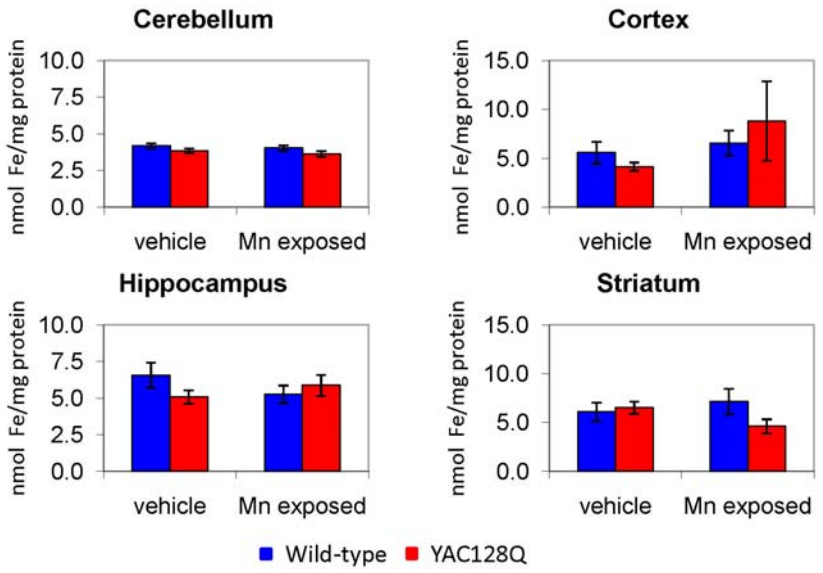


Figure IV.4. Fe Levels in Mn(II)-Exposed Animals. Wild-type and YAC128Q HD mice exposed to Mn(II) and Fe levels analyzed. ANOVA analyses see Table IV.2. *Post-hoc* analysis of the genotype effect found a significant difference in striatal manganese accumulation between wild-type and mutant animals as indicated (* $p<0.05$ *post-hoc t*-test).

Diminished Response of Fe Pathway to Mn in HD Model.

The levels of TfR, an essential component of the Fe signaling pathway, have been reported to be elevated in response to Mn intoxication and to be altered by mutant *HTT* (Kwik-Uribe et al. 2003; Simmons et al. 2007; Wang et al. 2008). Others have also reported elevated levels of this protein in the mutant ST*Hdh* cell line (Trettel et al. 2000). Analysis of protein expression levels by western blot indicated TfR levels were lower in the mutant cells when compared to the wild-type ST$Hdh^{Q7/Q7}$ cells with and without Mn exposure. After exposure to 40 µM Mn, a pathophysiological exposure, we observed the anticipated increase in the levels of TfR. However, no significant change was seen in the mutant ST$Hdh^{Q111/Q111}$ cell line after exposure to Mn, indicating decreased responsiveness to Mn exposure by the mutant ST$Hdh^{Q111/Q111}$ cell line as also observed for phosphorylated Akt (Figure IV.5. and III.8.-11.) (Williams et al. 2009).

Factor	Brain Region	F	Significance
Genotype	Cortex	$F_{(1,53)}$=0.12	0.73
	Striatum	$F_{(1,53)}$=1.636	0.207
	Hippocampus	$F_{(1,53)}$=0.425	0.517
	Cerebellum	$F_{(1,53)}$=5.45	0.024*
Mn	Cortex	$F_{(1,53)}$=3.099	0.085
	Striatum	$F_{(1,53)}$=1.201	0.278
	Hippocampus	$F_{(1,53)}$=0.118	0.732
	Cerebellum	$F_{(1,53)}$=3.192	0.08
Genotype x Mn	Cortex	$F_{(1,53)}$=1.25	0.269
	Striatum	$F_{(1,53)}$=3.262	0.077
	Hippocampus	$F_{(1,53)}$=2.163	0.148
	Cerebellum	$F_{(1,53)}$=0.291	0.592

Table IV.2.: Analysis of Regional Fe Levels by Multivariate 2-Way ANOVA. ANOVA analysis of total Fe levels showed a significant effect of Mn(II) exposure only in cerebellum ($p<0.05$ *post-hoc t*-test, not indicated).* significance at $p < 0.05$

Net Fe Accumulation is Altered Two Fold in the Presence of Mutant HTT.

Given the homeostatic relationships between Mn and Fe, we examined the effects of Mn on Fe levels in the ST*Hdh* cell lines (Chua et al. 1996; Garcia et al. 2006; Garcia et al. 2007; Fitsanakis et al. 2008). At lower concentrations, Mn had no effect on Fe levels in the striatal cells (Figure IV.6.A.; 0 and 40μM). However, at a higher exposure (100μM), we observed a significant increase in Fe levels in the wild-type cells that was not seen in the mutant cells (Figure IV.6.A.; 100μM).

Because of the altered Fe accumulation and the reduced TfR levels in the presence of Mn, we examined the effects of Fe exposure on net Fe levels in the ST*Hdh* cell (Figure IV.5.) (Williams et al. 2009). The addition of Fe had no significant effect on the two cell lines at lower Fe concentrations (Figure IV.6.B., 0 and 100μM). However, at 600μM, significantly higher Fe levels were observed in the ST$Hdh^{Q7/Q7}$ cells relative to the mutant cells (Figure IV.6.B.; 600μM). To understand the differences between Mn and Fe net accumulation in the cell model, we examined the fold changes between wild-type and mutant cells after exposure to either Mn or Fe. The maximal two-fold increase in net Fe uptake in wild-type cells relative to mutant *HTT* cells after Fe exposure was well below the almost ten-fold increase in net Mn uptake in wild-type cells relative to mutant *HTT* cells following Mn exposure (Figure IV.6.C.).

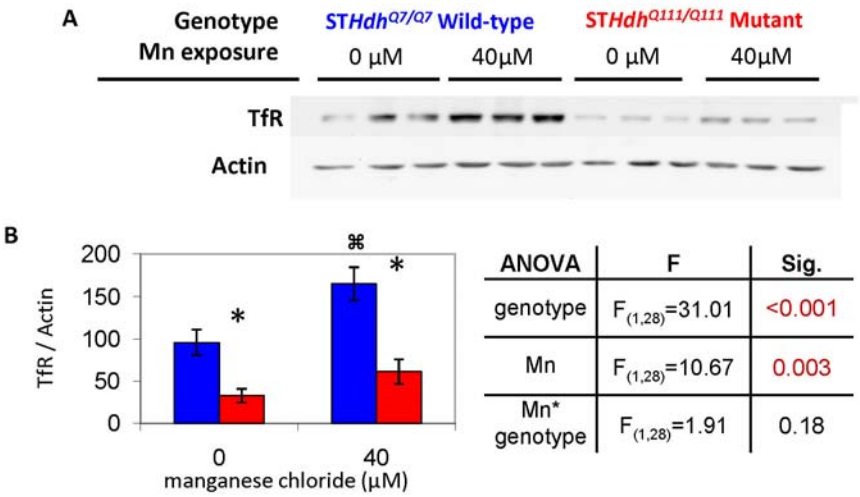


Figure IV.5. Mutant Cells have a Diminished Fe Response to Mn. Lysates harvested from ST*Hdh*$^{Q7/Q7}$ or ST*Hdh*$^{Q111/Q111}$ cell lines after 30 hours of manganese chloride exposure were analyzed by western blot for transferrin receptor (TfR), and Actin. (A) Representative blot showing 3 samples of each genotype-exposure group. (B) Quantification of protein expression graphed normalized to actin as a percent of the untreated by wild-type control (± SEM), averages represent independent samples n=7. Tables show statistical analysis from a two-way univariate ANOVA for each protein. Significant effects of genotype, exposure, or two-way interaction of genotype by exposure are designated by red numbering. * Significant difference in expression levels between genotypes at indicated exposure. ⌘ Significant difference of Mn exposure on protein expression level within wild-type genotype ($p<0.05$ *post-hoc t*-test assuming unequal variance).

Saturating Levels of Fe have No Significant Effect on the HTT-Mn Interaction.

To address more closely the role of the Fe transport and its potential contribution to the *HTT*-Mn gene-environment interaction, we examined the toxicity of Mn in the presence of saturating levels of Fe^{3+}, 600µM (Figure IV.7.). We reasoned that at high levels of Fe^{3+}, the TfR pathway would be saturated, limiting the transport of other metals through this pathway. Thus, we expected that high levels of Fe^{3+} would decrease the toxicity of Mn that is taken up by this Fe-saturable transport system, but would have a minimal effect on Mn toxicity driven by Fe^{3+}-independent uptake systems. Furthermore,

if changes in the activity of the Fe transport pathway substantially contributed to the changes in net Mn uptake in HD, then we would expect saturating levels of Fe^{3+} to diminish the *HTT*-Mn interaction. We found that saturating amounts of Fe diminish Mn toxicity in both cell lines (Figure IV.7.A.). A three-way univariate ANOVA of the data found a significant effect of genotype, Mn exposure, and a two-way interaction between genotype and Mn-exposure, recapitulating the gene-environment interaction (Figures II.4. and II.6.) (Williams et al. 2009). The ANOVA revealed no significant effect of Fe-exposure, or a two-way interaction for Fe-exposure by genotype, indicating no differential sensitivity to Fe^{3+} between the two cell lines. However, a two-way interaction between Fe-exposure by Mn-exposure was observed, demonstrating that Fe exposure reduces Mn toxicity. Importantly, the ANOVA found no significant three-way interaction of genotype by Mn-exposure by Fe-exposure. This indicates that while Fe reduced Mn toxicity, it did so equally between wild-type and mutant cells (Figure IV.7.). The equal response of the two cell lines in the presence of Fe can be seen when the data is regraphed to normalize out the toxicity of Fe (Figure IV.7.B.). Taken together these analyses strongly argue that defects in Fe^{3+}-saturable transport pathways are not sufficient to explain the HD-Mn disease-toxicant interaction.

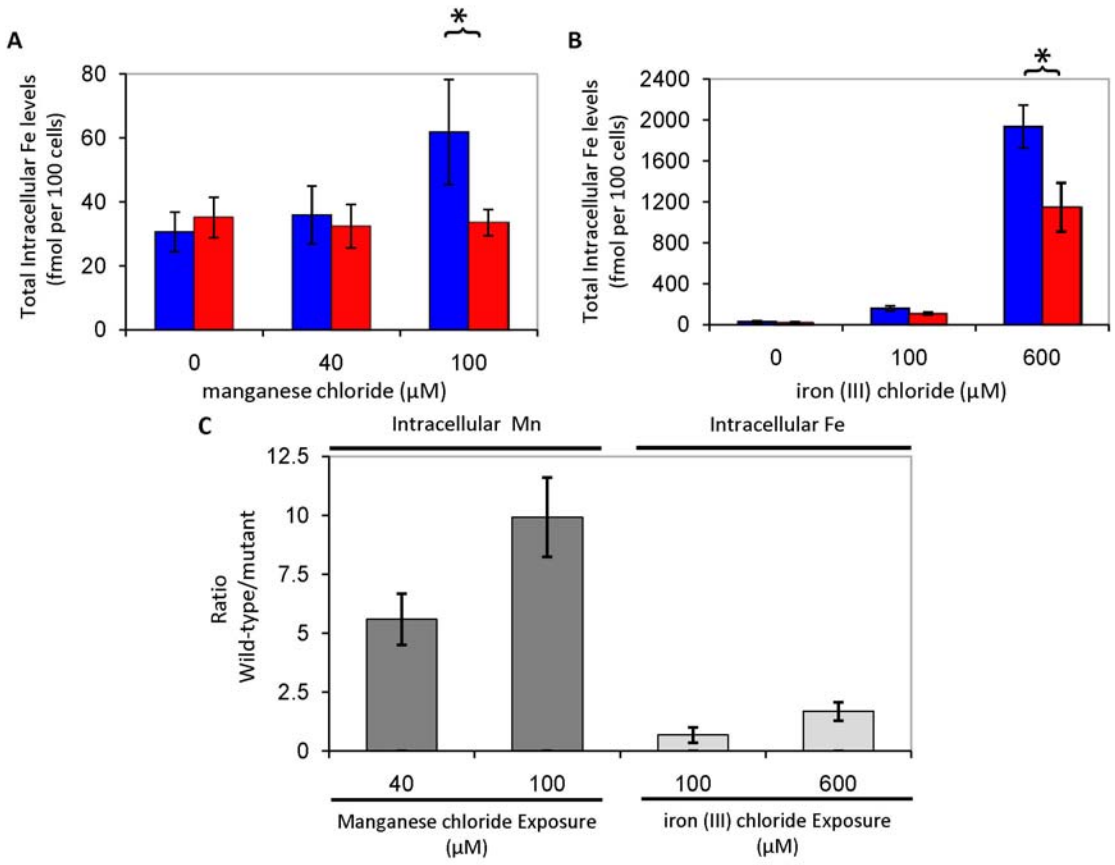
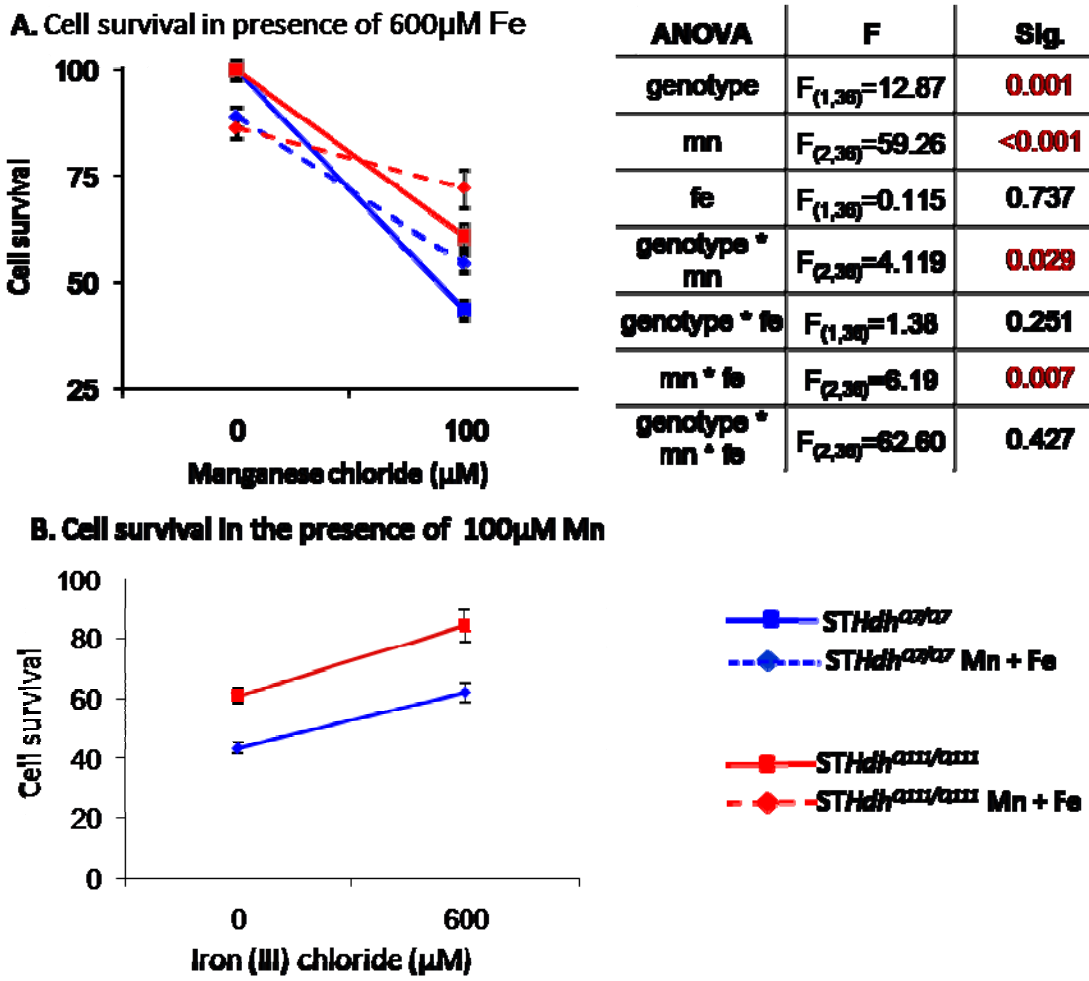


Figure IV.6. Fe Transport is Not Sufficient to Explain Mn Changes in the Presence of Mutant *HTT*. Measurement of total intracellular Fe levels in (Blue) ST*Hdh*$^{Q7/Q7}$or (Red) ST*Hdh*$^{Q111/Q111}$ cell lines after application of indicated concentrations of (A) Mn(II) chloride or (B) Fe(III) chloride for 30 hours. (C) Summary of data in Figure IV.1. and IV.6.B. expressed as the fold change normalized to mutant intracellular metal levels. n=5 independent experiments Error bars indicated the sum of normalized SEM for wild-type and mutant. * Significant difference in net metal uptake ($p<0.5$ *posthoc t-test* assuming unequal variance) between wild-type and mutant cells

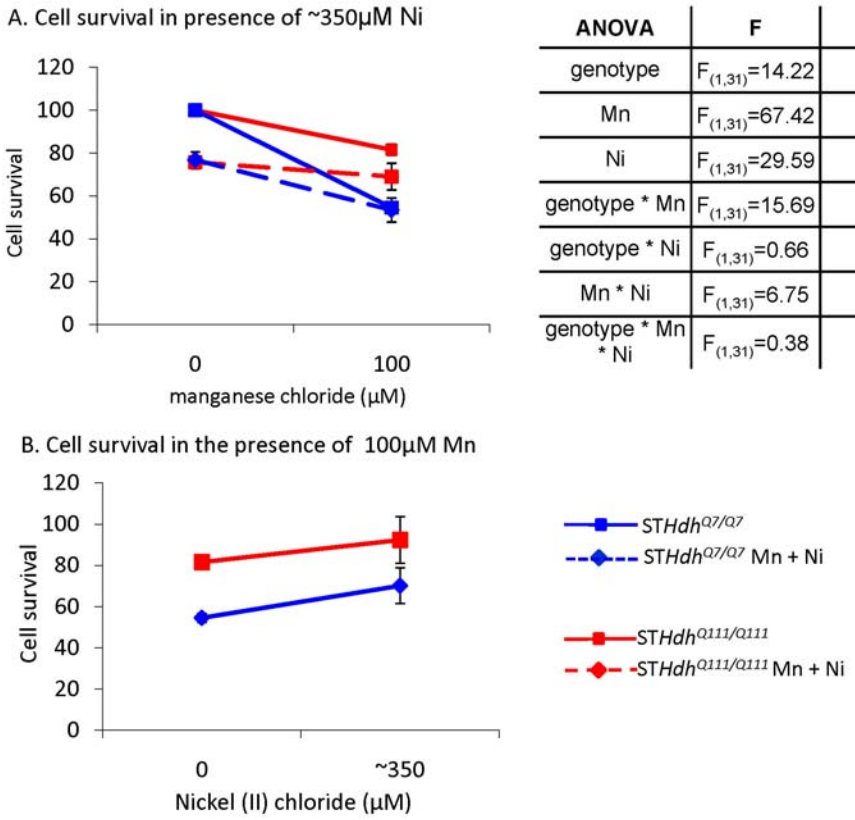


ANOVA	F	Sig.
genotype	$F_{(1,36)}$=12.87	0.001
mn	$F_{(2,36)}$=59.26	<0.001
fe	$F_{(1,36)}$=0.115	0.737
genotype * mn	$F_{(2,36)}$=4.119	0.029
genotype * fe	$F_{(1,36)}$=1.38	0.251
mn * fe	$F_{(2,36)}$=6.19	0.007
genotype * mn * fe	$F_{(2,36)}$=62.60	0.427

Figure IV.7. Saturating Levels of Fe Transport Reduce Mn Toxicity in Both Cell Lines. Measurement of cell survival in (Blue) ST*Hdh*$^{Q7/Q7}$or (Red) ST*Hdh*$^{Q111/Q111}$ cell lines after application of indicated concentrations of Mn(II) chloride (A) in the presence or absence of 600µM Fe(III) chloride. The average absorbance relative to the untreated control is graphed as percent untreated. (B) Survival Data normalized to control for Fe toxicity. All data points contain 100µM Mn (Note parallel lines). n=3 independent experiments with 4 independent wells each (± SEM). Table summarizes a three-way univariate ANOVA of this data. Significant effects for individual factors, two-way, or three-way interactions are designated by red numbering.

A High Level of Ni Does Not Affect the HD-Mn Interaction

The metal Ni can alter multiple pathways that are reported to transport Mn. Ni for example can block various calcium channels that allow Mn into the cell (Shibuya et al. 1992; Zamponi et al. 1996). Additionally, multiple divalent metals are transported by DMT1, including Mn, Cd and Ni (Gunshin et al. 1997). Our previous results demonstrated that Mn and Cd have opposite effects on cell survival in the presence of the HTT mutation (Figures II.3 and II.4.) (Williams et al. 2009). However, to explore the role of DMT1 and Ni blockable calcium channels in more detail we have analyzed the ability of Ni, which is also transported by DMT1 and has similar toxicity in both St*Hdh* cell lines, to alter Mn toxicity in the HD clonal cell line. We report that Ni does not alter Mn toxicity. Statistical analysis by ANOVA found significant effect of genotype, Mn, and Ni on the STHdh cell survival indicating that each of these factors alone alters the viability of the cells (Figure IV.8.A. and B.). Additionally, the analysis detected the HTT-Mn gene-environment interaction and a Mn-Ni interaction because of the ability of Ni to influence the toxicity of Mn. However, a three-way interaction was not reported, as Ni alters the sensitivity of both the cells to Mn (Figure IV.8.). The lack of significant effect of Ni and genotype is represented in the regraphed data normalized for the toxicity of Ni (Figure IV.8.B.). Taken together this data suggests that Ni does not influence the toxicity of the mutant cell lines differently than wild-type, limiting the Mn toxicity in both lines but more so in the mutant ST$Hdh^{Q111/Q111}$ line.



ANOVA	F	Sig.
genotype	$F_{(1,31)}$=14.22	0.001
Mn	$F_{(1,31)}$=67.42	0.001
Ni	$F_{(1,31)}$=29.59	0.001
genotype * Mn	$F_{(1,31)}$=15.69	0.001
genotype * Ni	$F_{(1,31)}$=0.66	0.426
Mn * Ni	$F_{(1,31)}$=6.75	0.016
genotype * Mn * Ni	$F_{(1,31)}$=0.38	0.542

Figure IV.8. High Levels of Ni Reduces Mn Toxicity in Equally in Both Cell Lines. Measurement of cell survival in (Blue) ST*Hdh*$^{Q7/Q7}$or (Red) ST*Hdh*$^{Q111/Q111}$ cell lines after application of indicated concentrations of Mn(II) chloride (A) in the presence or absence of ~350µM Ni(II) chloride. The average absorbance relative to the untreated control is graphed as percent untreated. (B) Survival Data normalized to control for Ni toxicity. All data points contain 100µM Mn (Note parallel lines). n=3 independent experiments with 4 independent wells each (± SEM). Table summarizes a three-way univariate ANOVA of this data. Significant effects for individual factors, two-way, or three-way interactions are designated by red numbering.

Discussion

The significant decrease in net Mn accumulation in the mutant cells justifies the strong Mn(II) resistance phenotype, and suggests a Mn homeostatic defect due to mutant *HTT*. Of particular note, a comparison of cell survival and total Mn accumulation reveals that wild-type cells exposed to 40 µM Mn(II) have statistically indistinguishable cell

survival and total Mn levels relative to mutant cells exposed at 100 μM Mn(II) (Figures II.4. versus IV.1.). These data strongly suggest that impairment in Mn accumulation may contribute, at least in part, to the Mn(II) resistance phenotype of ST*Hdh*$^{Q111/Q111}$ cells. Furthermore, the cellular disease-toxicant interaction accurately predicted a defect in Mn accumulation in the striatum of the YAC128Q HD mouse model. Indeed, the *in vivo* study showed a Mn-accumulation deficit in the very brain region most vulnerable in HD (Figure IV.4.).

Wild-type *HTT* function has been linked to TfR, a known Mn^{3+} transporter (Metzler et al. 2000; Lumsden et al. 2007). HD patient data have also shown important alterations in Fe signaling where both Fe levels and ferritin levels are altered (Dexter et al. 1991; Bartzokis et al. 1999). Only one study has examined Mn levels in HD brains, reporting no significant alterations in Mn levels; the study examined only four HD striatal samples and therefore is statistically under-powered to detect small effects (Dexter et al. 1991). Our study reported decreased Mn uptake after injection of the YAC128 mouse model of HD specifically in the striatum (Figure IV.4.) (Williams et al. 2009).

We report no significant changes in protein levels of the TfR in the mutant cell line after Mn exposure (Figure IV.5.). Additionally, examination of the fold changes in net metal accumulation between wild-type and mutant StHdh cells after exposure to Fe, display a maximal two-fold increase in net Fe uptake in wild-type cells relative to mutant *HTT* cells (Figure IV.6.A.). The two-fold change is much less than the almost ten-fold increase in net Mn uptake in wild-type cells relative to mutant *HTT* cells after Mn exposure we reported previously (Figure IV.6.B.) (Williams et al. 2009). Finally, exposure to Mn in the presence of saturating levels of Fe shows no significant effects on

the mutant HTT mediated Mn resistance phenotype (Figure IV.6.C.). We observed small alterations in Fe uptake in HD mutant cells (Williams et al. 2009). However, given that TfR has a higher affinity for Fe than Mn (Moos et al. 2000; Takeda et al. 2000), and the inability of Fe to block the HD-Mn interaction (Figure IV.7.). Our data suggest a minor role for Fe transport pathways in this interaction. We conclude that the Fe-saturable TfR pathway plays a limited role in the resistance to Mn toxicity caused by the HD mutation phenotype we reported previously.

Additionally, we examined the ability of Ni to alter Mn toxicity and found it does not block the toxicity more in the mutant line (Figure IV.8.). Therefore, the data suggests that a Ni blockable mechanism is not responsible for the HD-Mn phenotype we have found. Other Mn transport systems such as zip8, various calcium channels, as well as the ionotrophic glutamate receptors are other possible mechanisms of Mn entry that could be aberrant in HD (Au et al. 2008). Additionally, we have not explored the roles of storage or export in the reported reduction in Mn toxicity.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

It is clear that HD is a monogenetic disorder caused by an expansion of CAG tract within the *HTT* gene (Cowan et al. 2006; Imarisio et al. 2008). However, it has also become evident that the length of the polyglutamine repeat alone cannot account for all of the variability seen in disease progression, severity or the age of onset (Wexler et al. 1987; Wexler et al. 2004). Monozygotic twin studies have shown that even with the same genetic history, patients can present with different symptoms and at different ages emphasizing the role of the environment (Georgiou et al. 1999; Gomez-Esteban et al. 2007; Panas et al. 2008). For example, Friedman et al. found a potential role for both cigarettes and exposure to industrial toxins as a potential cause of discordance for HD in one twin study (Friedman et al. 2005). Environmental modifiers, such as exposure to drugs and pesticides, have been shown to lead to the progressive loss of the dopaminergic neurons of the substantia nigra and cause PD (Langston et al. 1999; McCormack et al. 2002). Exposures to metals such as copper are also considered a risk factor in the development of PD (Gorell et al. 1999). Additionally, Mn exposure is considered a risk factor for PD and the accumulation of the metal in the brain can lead to a PD-like disorder (Mergler et al. 1994; Gorell et al. 1999). The damage caused by metal exposure that increases the risk to neurodegenerative disease is associated with increases in

oxidative stress. Metals can increase lipid peroxidation and reduce the activity of antioxidant enzymes (Gaeta et al. 2005).

Interestingly, the first models of HD display the importance of gene-environment interactions, as the toxicant, 3NPA, a mitochondrial complex II inhibitor, was used to induce a striatal specific neurodegeneration creating a HD-like phenotype (Beal et al. 1993). The 3NPA model continues to be used today in the study of HD and has been shown to cause mitochondrial dysfunction, oxidative stress, and excitotoxicity; and alter metal homeostasis pathways. Each of these pathways has been shown to be changed due the HD mutation and is implicated as a primary insult in the pathology of HD (Dexter et al. 1991; Beal et al. 1993; Imarisio et al. 2008). Based upon this toxicant interaction with HD, we proposed the following hypothesis: That neurotoxicants sharing primary common pathophysiological targets with mutant HTT will display gene-environment interactions. The use of the toxicant as a tool to identify cellular functions of the HD mutation is novel. It is not necessary that HD patients would be exposed to these toxicants as a part of normal disease progression for us to exploit their ability to modify the disease.

Toxicants should influence the known biology of the disease to test our hypothesis. A hallmark of HD biology is the selective degeneration of the basal ganglia, which leads to the characteristic HD chorea. Metal toxicity can also produce regional cell loss in the brain. In the case of Mn, the globus pallidus accumulates the metal leading to a hypokinetic disorder similar to PD (Yamada et al. 1986; Olanow et al. 1996; Olanow 2004; Fitsanakis et al. 2006). Additionally, like 3NPA and HD, Mn

overexposure has been shown to alter mitochondrial function, increase reactive oxygen species, excitotoxicity, and alter the levels of Fe within the brain (Aschner et al. 1990; Gavin et al. 1990; Gavin et al. 1999; Zhang et al. 2004; Garcia et al. 2007; Milatovic et al. 2007).

Using metals for our novel screen, we discovered two previously unreported interactions with the HD mutation. First, sensitivity to Cd toxicity is increased in the presence of the polyglutamine expanded form of HTT. Secondly, sensitivity to Mn toxicity is reduced in the presence of mutant HTT. This surprising result is the first reported where full length HTT polyglutamine expanded improves the viability under any condition, while many stressors have been shown to increase cell death. Some exon 1 HTT constructs have been shown to limit excitotoxicity, an activity ascribed to the proline rich domain of HTT. The neuroprotective effect is hypothesized to occur only in these short HTT constructs because normal folding of the protein may block these interactions (Zuchner et al. 2008). The ST*Hdh*$^{Q111/Q111}$ cells have increased cell death when exposed to 3NPA; under conditions that increase excitotoxicity; or when extra-mitochondrial energy or mitochondrial energy is altered (Trettel et al. 2000; Gines et al. 2003; Gines et al. 2003; Seong et al. 2005; Milakovic et al. 2006; Oliveira et al. 2006; Lee et al. 2007).

The Phenotype Differences of the ST*Hdh* Cell Lines in the Absence of Mn

The mutant and wild-type mouse ST*Hdh* cell lines were made from a wild-type or knockin mouse model of HD (Cattaneo et al. 1998; Trettel et al. 2000). Because these cells were created from different animals, it was possible that differences related to Mn

we observed were due to inherent differences in the lines not directly related to the expression of the mutation (Figure II.4-6.). We found differences in cell division rate, cellular LDH activity, and Akt phosphorylation in the absence of Mn exposure, characteristics of these cells which have also been published by others (Figure II.1, 2, 8, and 9.) (Trettel et al. 2000; Gines et al. 2003). The reduced cell division rate exhibited by the mutant line has been attributed to the 'immortalization' of the cells by forced expression of SV40 large T antigen. The SV40 large T antigen blocks the activities of p53. Levels of this key regulator of cellular stress and apoptosis are also reduced in the mutant STHdh cell line (Trettel et al. 2000). Basal levels of the serine/threonine protein kinase B or Akt are elevated in the ST*Hdh*$^{Q111/Q111}$ line when compared to wild-type (Gines et al. 2003). Akt is a key regulator of neuronal survival and its phosphorylation at S-473 can be increased by exposure to Mn and other metals (Bae et al. 2006). Closer examination of the Akt pathway in the STHdh cells showed that Akt was responding to increased calcium flow via the NMDAR (Gines et al. 2003).

HTT is directly phosphorylated by Akt and the phosphorylation can improve cell viability in models of HD that over express the N-terminus of HTT (Saudou et al. 1998; Humbert et al. 2002). The effect of Akt phosphorylation on survival in a full-length HTT model is still unknown. However, it is difficult to know the role of these differences in the HD-Mn interaction by comparing only these two cell lines. Therefore, we have examined the HD-Mn interaction using both transfection and primary culture models. We found that the mutant HD protein limits Mn toxicity in every system we tested (Figure II.2-6, III.3, 6, and IV.3.). Our data also indicates that over-expression of the mutation in cells already containing wild-type HTT can confer the resistance to Mn,

suggesting that a novel gain of function effect on the mutation may be the source of the phenotype (Figure III.3.).

High Levels of Mn Increases Aggregation Rates of Ataxin 1

One hallmark of multiple neurodegenerative diseases is the formation of protein aggregates in patients. Aggregates form in HD patients and in some mouse HD models (Ross et al. 2004). Full-length HTT will not readily aggregate in a cell culture model. Therefore, we used a different polyglutamine expanded protein, Ataxin 1, to analyze the role of Mn in polyglutamine-dependent aggregate formation, this adds to the complexity, as the survival and aggregate data cannot be correlated. Ideally, this study should be repeated in a polyglutamine model that displays both aggregates and the gene-environment interaction. Importantly, similarities do exist between Ataxin and HTT. Both of these polyglutamine expanded proteins are believed to undergo a similar process of aggregation (Uversky et al. 2001; Masino et al. 2002; Ross et al. 2004; Bhattacharyya et al. 2005). Our experiment did address the ability of Mn to induce microscopically visible aggregates. We discovered that Mn increases the aggregation or Ataxin 1 (Figure II.8.). Currently the implication of visible aggregates is debatable. Soluble HTT aggregates are now emphasized as a toxic species, as some believe that these late stage visible aggregates have a protective role in the cell (Kuemmerle et al. 1999). Regardless, the levels of Mn necessary for us to see a significant effect on protein aggregation are much higher (500 μM) than the levels necessary to see effects on survival (40 μM) (Figure III.8.). It is possible that lower levels of Mn are affecting aggregates not detected

under these conditions but could affect cell survival. The difference in these concentrations suggests that the formation of visible aggregates is not directly related to the cell survival differences.

The HTT Alters Sensitivity to Mn in Multiple Models of HD

Our transfection studies not only allowed confirmation of the interaction between Mn and HTT in a single cell line, but they also allowed us to define the interaction further. Our data indicates that the first third of the HTT protein, which contains the polyglutamine domain, is sufficient to reduce Mn toxicity. The first third of HTT is still a very large segment of the protein, containing approximately 1200 amino acids. Additional studies will allow us to determine the essential regions of HTT needed for this interaction. The polyglutamine region may not be the only region essential for the phenotype. Expansion of the glutamine tract could lead to a conformational change in the protein and interfere with protein interactions that occur in another region of HTT. For example, near the polyglutamine domain of HTT there is a proline rich domain, which is thought to add in protein solubility (Dehay et al. 2006). The proline rich domain is also believed to mediate cell survival after excitotoxic insults in exon 1 models of HTT (Zuchner et al. 2008). Discovery of the minimal regions of HTT required for the alterations in Mn uptake will be important future studies. Identification of the minimal region will allow us to determine if HTT and the transporter directly interact for example. Determining the region of HTT required for the phenotype would be paramount to

understanding if the interaction is caused or lost by the glutamine expansion indicating a possible a gain of function or loss of function mutation.

The primary culture experiments were crucial in determining that the phenotype existed in other models of HD and in these cells, we see the ability of HTT to limit Mn toxicity (Figure III.6.). Our primary culture data is largely from cerebellar cultures. Granule neurons are the predominate cell type in the cerebellum and these cells are GABAergic. Given that medium spiny neurons of the striatum are also GABAergic, these cultures are highly relevant. The NMDAR has been implicated in the striatal specific death associated with HD (Beal et al. 1986; Greene et al. 1993) and the medium spiny neurons express both the NR1A and NR2B subunits of the NMDAR (Landwehrmeyer et al. 1995; Kuppenbender et al. 1999). Cerebellar granular neurons express NR2B neurons in culture and *in vivo* initially before maturing (Vallano et al. 1996; Kovacs et al. 2001). Additionally, the NMDAR has been linked to Mn toxicity (Brouillet et al. 1993). Therefore, the persistence of the Mn phenotype in these cultures may implicate the role of this receptor. In both HD and Mn overexposure, the NMDAR is associated with excitotoxicity. HD models show increased sensitivity to excitotoxic stressors and but not to Mn implicating another pathway in the HD cell loss (Figure II.4-6.). Additional studies in a neuronal model that does not express the NR2B subunit, perhaps cerebellar cultures from older animals, would shed more light on the role of this receptor subunit on the phenotype. Future studies could also directly inhibit the NMDAR to examine its role in the Mn resistance phenotype.

To date, striatal cultures have proven challenging and this has limited the number of questions we were able to answer. We utilized a culture from the YAC128 animal that was created using a yeast artificial chromosome expressing the HD mutation. We cultured cells from each animal separately because of the lack of a rapid genotyping procedure. Plating a small number of cells in each dish can limit the viability of primary cultures. To improve these cultures, we propose two methods (1) use homozygote *HTT* knock-in mice crossed to wild-type animals so that cultures could be combined without genotyping or (2) try embryonic cultures that are reported to increase viability.

Role of Cell Type in HTT-Mn Interaction

One of the most important questions we could ask utilizing the primarily culture model is the role of different cell types in the HD-Mn interaction. Our current data suggests that neurons may be more sensitive but staining of these cultures must be done to determine the types of cells present before any conclusions can be made (Figure III.6. and 7.). The variability of the brain environment is complex consisting of multiple regions and cells each with diverse connections and signaling molecules. Understanding the role that different cell types and perhaps regions play in this interaction will be necessary to understand the process of selective degeneration.

We report a reduction in cell viability at concentrations of Mn as low as 40 μM in the wild-type cells (Williams et al. 2009) (Figure II.4. and 6.). Another group reported similar levels of toxicity in neuronal cultures (Malecki 2001). Astrocytes, however, have

a greater resistance to Mn induced toxicity with significant levels of cell death at 250 μM (Lee et al. 2009). The reduced toxicity of Mn in astrocytes reflects their ability to accumulate large quantities of Mn both in the brain and in culture. These cells accumulate 10-50 fold more Mn than neurons. Mn is needed to ensure proper function of the astrocytic enzyme GS (Wedler et al. 1984).

Human brain levels of Mn are approximately between 0.5-4.0 μg/g-wet tissue weight depending on the region (Yukawa et al. 1980; Bonilla et al. 1982; Bush et al. 1995; Tracqui et al. 1995). After exposure to Mn, striatal Mn levels can increase up to 12 fold and the levels in the globus pallidus 9-fold in the monkey (Suzuki et al. 1975). Mn levels in the brain are also altered due to the levels of other metals. Decreased Fe levels specifically have been shown to increase Mn uptake (Erikson et al. 2006). Alternatively, Ca influx has been shown to reduce cellular levels of Mn in glia and astrocytes (Wedler et al. 1994).

Energy state of the brain can also alter the levels of various metals because some metal transporters (e.g. Cu-ATPase) require energy for proper function (Bush 2000). Both reduced energy homeostasis and increased levels of Fe in the striatum are associated with HD and may play an essential role in the uptake deficit before and after exposure to Mn. Due to the effects that other metals have on Mn accumulation, it is important to note the concentrations in our culture media. Only the levels of Ca^{+2}, Mg^{+2}, Fe^{+3}, potassium, and sodium are in significant levels in the DMEM we use for cell culture. The inorganic salt in highest concentration is sodium (~167mM). Ca and Fe concentrations are 2.4mM

and 0.2 μM respectively. Because both cell lines are cultured in the same media, this cannot explain the difference between the mutant and wild-type cell lines.

Mn Transport Deficiency in HD Models

The inability of Mn to induce Akt phosphorylation in the ST*Hdh*$^{Q111/Q111}$ cells led us to the discovery of the Mn accumulation deficit (Figure III.8, 9, and IV.2.). Most importantly, upon exposure to Mn there is a deficit in Mn accumulation in the YAC128 animal model and validates our initial hypothesis: A toxicant sharing pathophysiological mechanisms with HD exhibits disease-toxicant interactions (Figure IV. 3.). We have yet to elucidate the mechanisms of toxicity reflected in this interaction through our transport studies but analysis of these pathways has indicated that both DMT1 and the TfR pathway are not the source of the interaction (Figure II. 4, and IV.4.-6). Cells cultured from the Belgrade rats that have a mutation in DMT1 transfected with mutant HTT could help elucidate the role of this Mn transporter in the phenotype (Fleming et al. 1998). A pilot study suggests that Ni block able calcium channels do not play a role in the HD-Mn phenotype (Figure IV.8.). Unfortunately, because Ni also is toxic to the cells the interpretation of the data is extremely complex; use of specific channel inhibitors if available would allow us to precisely examine the roles of these channels. Additional studies will focus on other transport mechanisms such as the Zip8 family and calcium channels. Finally, other mechanisms that would alter cellular Mn levels such as export and storage must be examined.

Akt Signaling in HD

Akt phosphorylation at S473 is associated with neuroprotection and cell viability. In our cell model, phosphorylation at this residue is increased in the mutant cells without the addition of Mn (Figure III.8. and 9.) (Trettel et al. 2000; Gines et al. 2003; Williams et al. 2009). The elevated Akt activity in the presence of mutant HTT is downstream of the NMDA receptor, which is linked to the heightened excitotoxicity seen in these cells and the mitochondrial defects (Gines et al. 2003). Interestingly, we discovered an inability of the mutant cells to respond to Mn via Akt phosphorylation while they robustly respond to Cd (Figure III.8.). Currently, Akt activation by metals is thought to be via the creation of ROS (Lopez et al. 2006; Liao et al. 2007). Concentrations of Mn above 100 μM in the ST*Hdh*$^{Q111/Q111}$ cells may be activating Akt in a similar manner to Cd exposures perhaps via ROS. However, both cell survival and Akt response is very different at low exposures of Mn (40 μM and below) indicate a separate pathway of activity (Figure II. 6, III.8. and 9.) (Williams et al. 2009). Akt can also be activated by phosphorylation at threonine 305, which increases its activity. Currently, it is not known how mutant HTT or Mn alters the phosphorylation of Akt at Threonine 305. Examination of the phosphorylation site may reveal multiple levels of Akt activity and explain the different responsiveness of the ST*Hdh*$^{Q111/Q111}$ cells to various Mn concentrations.

HD Mutation Alters Mn Accumulation

Our data also indicates a deficiency in Mn in the ST*Hdh*$^{Q111/Q111,}$ which is reflected in the apparent increased survival at low concentrations of Mn (Figure II.7. and IV.2.). Although we did not detect Mn deficiency in the presymptomatic HD mouse model, it is possible such a deficiency would arise with disease progression (Figure III.3.). The YAC128 model used in this study were injected at 3 months and the first symptoms do not appear until 9 months. It is also possible that a Mn deficiency would be limited to one cell type limiting detection in the dissected brain regions. For example, Mn accumulates in astrocytes predominately which play a critical role in neuronal survival by providing neurons with both glutamine and lactate for energy. Astrocytes, which accumulate the majority of Mn, could be deficient in Mn leading to a reduction in energy provided to the neurons. Surviving neurons could then mask any deficiency in Mn until late in disease after they have perished due to the reduced energetic substrates. Examination of energy and mitochondrial respiration in the StHdh lines reported that respiration was similar in both cell lines. However, ATP levels were reduced when glycolysis is inhibited suggestive of an energy defect upstream of the mitochondria (Milakovic et al. 2005)

There are few reports on the molecular consequences of Mn deficiency in adult animals, but Mn is required for the proper function of many enzymes key to brain function (e.g. glutamine synthetase (GS), pyruvate decarboxylase, superoxide dismutase 2 (SOD2), arginase, and serine/ threonine protein phosphatase) (Applebury et al. 1970;

Greger 1999; Ensunsa et al. 2004; Eid et al. 2008). Menke's disease is associated with a deficiency in the metal Cu (Bertini et al. 2008). Cu, like Mn, is essential and incorporates into multiple metalloenzymes. Cu dependent enzymes (e.g. dopamine β-hydroxylase, cytochrome c oxidase, and ceruloplasmin) have reduced activities in Menke's disease (Kodama et al. 1999). The current treatment for this disorder is intravenous supplementation of Cu though its success is limited (Kaler et al. 2008).

Several Mn-dependent enzymes have direct links to HD. The activity of GS, which metabolizes glutamate, an excitatory neurotransmitter, to glutamine, is decreased in the corpus striatum of HD patients (Carter 1981; Butterworth 1986). GS is predominately expressed in astrocytes. Importantly, other glial-specific activities are elevated in HD, suggesting that GS activity is not lost due to general glial cell loss (Butterworth 1986). A reduction in GS activity is also associated with increased excitotoxicity, an established mechanism for HD-mediated cell death (Zeron et al. 2001; Fernandes et al. 2007). Expression of mutant *HTT* has been reported to increase sensitivity of astrocytes to excitotoxicity (Shin et al. 2005). Additionally, cells expressing the HD mutation show increased sensitivity to 3NPA (Figure II.3.) (Andreassen et al. 2001; Ruan et al. 2004). Deficiencies in SOD2 that is essential to limiting oxidative stress within the cell have been shown to lead to an increased sensitivity to 3NPA, a toxicant that has been used to model HD (Beal et al. 1993; Andreassen et al. 2001).

Altered energy metabolism has been reported in the ST*Hdh*$^{Q111/Q111}$ cell line. Interestingly, decreased energy metabolism is also reported in the HD patient. HD patients often suffer from diabetes and low body weight, directly linked to a decrease in

insulin production (Ye et al. 2009). Dietary restriction has even been shown to improve symptoms in an HD mouse model (Duan et al. 2003). It is intriguing that Mn has been reported to increase the activity of insulin degrading enzyme, which leads to improved insulin secretion (Baly et al. 1984). It is also important to note that insulin signaling in brown adipose tissue regulated by the protein Akt, where Akt stimulates the uptake of glucose (Hernandez et al. 2001). The urea cycle is deficient in HD patients and HD mouse models. Arginase regulates the final step of the urea cycle (Stoy et al. 2005; Chiang et al. 2007). Arginase activity has been linked to neuron survival by the reduction of nitric oxide signaling (Estevez et al. 2006). In symptomatic HD animal models, nitric oxide activity is increased, specifically in the striatum (Deckel et al. 2002). Thus, alterations in Mn levels have the potential to affect many different enzymes, which could explain the diversity of pathophysiological effects seen in HD. Importantly, improvement in the function of these enzymes could increase the survival of cells expressing the HTT mutations and we do see increased survival at low concentrations of Mn. We therefore must examine the function of these and other Mn-dependent enzymes in various models of HTT.

The primary neuronal cultures were done from cerebellum of postnatal day 0 to 1 pups and mutant cell display the resistance to Mn when compared to wild-type cells (Figure III.6.). However, we do not detect a deficiency in Mn accumulation in this brain area in our animal injection studies (Figure IV.3.). The data strongly indicates the role for brain environment and emphasizes that metals do not accumulate equally throughout the brain. Perhaps the striatum accumulates metals because of an intrinsic need of the region. Mn overexposure alters protein expression of various Mn transporters in the

brain but no studies indicate the effect of a deficiency (Garcia et al. 2006). TfR levels are altered in the ST*Hdh*$^{Q111/Q111}$ (Trettel et al. 2000; Williams et al. 2009). We do not know about the levels of other Mn transporters.

Given the potential for defects in Mn homeostasis to contribute to HD, modulation of Mn exposure has potential clinical importance for the HD patient. In the ST*Hdh*$^{Q111/Q111}$ cells, we see improved cell survival with low levels of Mn exposure (Figure II.7.). Our *in vivo* data show a specific effect on striatal Mn accumulation, indicating a possible role for altered metal accumulation in creating the selective degeneration pattern found in HD (Figure IV.3.). However, effective treatment of metal homeostasis disorders is especially complex, as indicated by the tightly regulated relationship between Fe and Mn, as well as the challenges in treating other metal disorders such as Menke's and Wilson's diseases (Chua et al. 1996; Madsen et al. 2007; Fitsanakis et al. 2008). These diseases result from mutations in two related genes, *ATP7A* and *ATP7B* respectively, which both encode copper transporters. In Wilson's disease, *ATP7B* mutations leads to an accumulation of copper while in Menke's there is a deficiency in Cu uptake due to changes in *ATP7A* (Ala et al. 2007; Bertini et al. 2008). The impact of mutant *HTT* may be both regional (striatal vs. cerebellar) and cell type-specific (neuronal vs. glial). General supplementation may increase Mn levels in areas that are not deficient, leading to toxicity without reaching therapeutic level where needed. Thus, future studies are needed to determine the mechanisms of the Mn transport deficiency and its potential role in selective pathology before we can understand the therapeutic potential of metal supplementation in HD. Collectively, these findings identify a novel environmental agent that may influence HD. Our data also shed light on

previously unexplained published work finding deficits in Mn dependent enzymes in HD patients and animal models.

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