A Thesis Entitled

The Evaluation of Neurotrophic Factor's Ability to Prevent Induced Cell Death in a PC12 Cell Based Huntington's Disease Model

by

Alexander S. Wisner

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Pharmacology and Toxicology

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An Abstract of

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Huntington's disease (HD) is caused by a genetic mutation of the IT-15 gene resulting in the expansion of the trinucleotide CAG in the huntingtin protein (MacDonald et al., 2003). The mechanism(s) by which this expanded protein causes the disease remains unknown, and there is currently no treatment to halt or prevent development of the disease. An assay was designed to measure the ability of three neurotrophic peptides (Colivelin, D-SAL, D-NAP) to protect cultured PC12 cells from death caused by an expanded form of human huntingtin exon 1. Each neurotrophic peptide was tested at six concentrations for its ability to protect cells against huntingtin-induced cell death. Cell viability was determined by an LDH activity assay that measured the change in absorbance over time. Neurotrophic peptides Colivelin and D-SAL promoted cell viability in both treatment types tested whereas D-NAP only promoted cell viability in the twenty-four hour pre-incubation treatment type.

Dedicated to the two people that have always been understanding and compassionate with me through the highs and the lows, my mother Kristina Wisner and father Stephen Wisner. I am only now starting to understand all the sacrifices you have made for me and they are truly appreciated. That being said I will still never admit you were right about anything!

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Table of Contents

List of Figures

Chapter 1

Introduction

1.1 Huntington's Disease Overview

1.1.1 Polyglutamine Diseases as a Group

A genetic mutation causing expansion of trinucleotide repeats was first identified in 1991 as the causative mechanism of spinal and bulbar muscular atrophy and fragile X syndrome (Koshy and Zoghbi, 1997). This genetic defect is caused by the inheritance of unstable DNA that alters the total number of trinucleotide repeats between each generation (Koshy and Zoghbi, 1997). The expansion of the total number of trinucleotide repeats leads to a phenomenon seen in polyglutamine related diseases and is referred to as anticipation. Anticipation is the increase in severity of the disease with earlier age of onset of symptoms in each successive generations (Koshy and Zoghbi 1997).

There are currently four types of trinucleotide repeat expansions that have been identified: long guanine-adenine-adenine (GAA), long cytosine-thymine-guanine (CTG), long cytosine-guanine-guanine (CGG), and short cytosine-adenine-guanine (CAG). Huntington's disease (HD) is one of eight known inherited neurodegenerative diseases that are caused by CAG expansion tracts in eight unrelated proteins (Ho et al. 2001). The CAG repeat expansion is located in the coding region of each respective gene and then translated into consecutive glutamine residues in each of these eight unrelated proteins. Despite these proteins being ubiquitously expressed throughout peripheral and nervous tissue only select groups of nerve cells are susceptible to degeneration.

1.1.2 Genetics of Huntington's Disease

The gene and its mutation were first identified in 1993 by the Huntington's Disease Collaborative Research Group (Ho et al. 2001). The HD-causing gene was named IT-15 for "interesting transcript number 15", being the 15th gene sequence examined from the predicted region (Nance 1998). The IT-15 gene is located on the short arm of chromosome 4 (4p16.3) spanning 210 kb and encodes a 10-11 kb transcript that is ubiquitously expressed (Koshy and Zoghbi 1997). This transcript then encodes a novel 350 kDa protein termed huntingtin (Htt) whose exact function remains to be determined.

The wild-type IT-15 gene has an uninterrupted CAG trinucleotide repeat lengths ranging from 10 to 35 and is translated into a polyglutamine (polyQ) tract near the N-terminus of the protein (Nance 1998). Individuals with CAG repeats in excess of 35 are considered to have the HD form of the IT-15 gene. Both the wild-type and mutant forms of the alleles are transcribed and are electrophoretically distinguishable by Western analysis (Koshy and Zoghbi 1997). On western blots the normal protein is seen as the expected 350 kDA band whereas the mutant forms migrate at a size dependent upon the total length of the expanded polyQ region (Koshy and Zoghbi, 1997).

HD exhibits an autosomal dominant mode of inheritance, meaning each child of an affected person has a 50% chance of inheriting the diseased gene (Nance 1998). Mutations of the IT-15 gene occur in 1% to 3% of individuals with less stable forms expressing 29-35 CAG repeats (Ho et al., 2001). Individuals with the upper limit of the normal range of CAG repeats are likely to die before the onset of symptoms and are thought to represent the pool of chromosomes from which the pathogenic "founder" mutations are derived (Ho et al., 2001). The IT-15 gene tends to increase the number of CAG repeats in succeeding generations. The highest frequency and degree of expansion is experienced as the IT-15 gene passes through male meiosis (Nance, 1998).

1.1.3 Huntington's Disease Pathogenesis

HD is defined as a genetic disorder characterized by programmed premature death of localized nerve cells and is viewed as a time dependent disease cascade. Despite the ubiquitous expression of Htt and mutant huntingtin protein (mHtt) only neurons of the brain are affected; other tissues of the body are either spared or do not manifest the disease. (Martin and Gusella 1986). The most severely affected neurons are in the striatum primarily composing the caudate and putamen (Martin and Gusella, 1986). There are five types of neurons in the normal human striatum: two kinds of medium-sized spiny neurons, one with abundant spinous processes (Type I) and one with sparse spiny processes (Type II); two kinds of aspiny cells, medium and large sized; and small neurons with variable dendritic morphology (Martin and Gusella, 1986). Approximately 80 percent of all striate neurons are the medium-sized spiny (Type I) and are the primary targets of HD (Holtser-Cochav et al., 2006).

Neuronal loss appears to proceed in a dorsocaudal to rostroventral gradient, with the most rostral portion of the caudate, the nucleus acumbens, generally showing the least cell loss (Nance, 1998). Pathologic changes in these cells consist of curling, branching, and arborizing of the dendrites. Indentations of the nuclear membrane, disorganization of the nucleolus and depletion of the ribosomes of rough endoplasmic reticulum have also been

found, with the degenerative changes in striatal neurons similar to those found in the cortex (Hickey and Chesselet, 2003). A neuropathological grading system devised by Vonsattel and others is commonly used (Hickey and Chesselet, 2003). The scale rates the macroscopic and microscopic appearance of the striatum and includes 5 "grades," from 0(normal) to 4 (severe atrophy) (Nance, 1998).Onset of neurological motor symptoms is estimated to occur when about 30 percent of neurons in the caudate and putmen are lost and the remaining cells are presumably dysfunctional (MacDonald et al., 2003). The identification of separate cell types and more than 30 possible neurotransmitters in the striatum has inspired extensive studies designed to characterize the cell types that appear to degenerate in HD and to correlate their function with the neurotransmitters they contain (Martin and Gusella, 1986).

The striatum receives a convergence of inputs from the cerebral cortex, thalamus, and brain stem. The input from the brain stem includes dopaminergic inputs from the pars compacta of the substantia nigra and noradrenergic and serotonergic inputs from the locus ceruleus and raphe nuclei, respectively (Martin and Gusella, 1986). Striatal outputs are relayed to the inner and outer segments of the globus pallidus and to the pars reticulata of substantia nigra (Martin and Gusella, 1986). The globus pallidus output also includes a loop to the subthalamic nucleus. The inner pallidus relays to the ventralis anterior and lateralis of the thalamus, which then

connect back to the cerebral cortex, completing the motor-control loop of the extrapyramidal system (Martin and Gusella, 1986). It is thought dopamine, glutamate and GABA are to be the most affected in HD along with other neurotransmitters and their corresponding receptors (Holtser-Cochav et al., 2006). Postmortem studies of the brain tissue from patients who had HD revealed the levels of many neurotransmitters, biosynthetic enzymes, and receptor-binding sites to be diminished or at abnormal levels (Martin and Gusella, 1986). The main excitatory neurotransmitter in the corticostriatal system is glutamate and activity of glutamic acid decarboxylase has been found to be reduced by 85 percent in the striatum and by 60 percent in the substantia nigra, putamen, and globus pallidus (Martin and Gusella, 1986).

1.1.4: Epidemiology

Huntington's disease has been described in virtually every major ethnic and racial groups (Nance, 1998). Both men and women are affected equally and typically become symptomatic in the third and fourth decades of their lives. There is a lack of widespread epidemiological studies of HD in the United States but it is estimated that approximately 25,000 – 30,000 individuals have manifested HD and a further 150,000 – 250,000 individuals are at risk for HD (Holtser-Cochav et al., 2006). There are large populations of patients with HD in Scotland and the Lake Maracaibo region of Venezuela

region. These areas exhibit a much higher frequency of the disease due to a founder effect in a physically or socially isolated community (Nance, 1998).

1.1.5 Clinical Aspects of Huntington's Disease

HD is diagnosed based on the combination of motor, behavioral and cognitive symptoms with a positive family history of the disease. DNA testing is used to show abnormal CAG expansion of the IT-15 gene and provide a confirmation of the diagnosis. Patients who have 40 or more copies of the gene will inevitably express the disease clinically and patients with a CAG repeat length in the range of 36-39 are at risk of expressing HD like symptoms (Holtser-Cochav et al., 2006). The life expectancy of individuals expressing symptoms of HD is approximately 15-20 years after the age of onset, which is typically ranges between 35 to 40 years of age. Cause of death is typically related to complications of immobility such as skin breakdown, pneumonia, cardiac disease or infection rather than HD itself (Holtser-Cochav et al., 2006). However, 25 percent of patients attempt suicide, and this is the cause of death in 8-9 percent of patients. HD typically exhibits an array of movement, cognitive, and psychiatric disorders.

Motor disturbances of HD are composed of two primary part: the presence of involuntary movements and the disturbance of voluntary movements (Nance, 1998). Chorea is usually considered the first sign of HD as it is the most outwardly noticeable aspect of the disease; however, the

impairment of voluntary movements may be far more functionally disabling than the chorea itself. HD chorea exhibits involuntary movements that are arrhythmic and irregular (Holtser-Cochav et al., 2006). Chorea worsens in the mid-stages of the disease and may become a safety issue involving larger amplitude movements causing injury or malpositioning. Frequent movements resulting in skin injuries, infection or even fractures and head trauma are also possible (Holtser-Cochav et al., 2006). The involuntary movements parallel the cognitive disorder in that the primary deficits relate to planning, sequencing, executing, and completing tasks (Nance, 1998).

HD can be categorized as a "subcortical dementia" to distinguish it from the "cortical dementias" of which Alzheimer disease is categorized. The most prominent symptoms include impaired judgment and executive function-that is, the inability to initiate, sequence, or complete a problem or task and difficulty with cognitive tasks that require sustained attention, mental flexibility, or speed (Nance, 1998). The dementia progresses over time, from visuospatial difficulties, nondominant hand slowing, impaired verbal learning, and disabled retrieval of learned information to a profound global dementia affecting all areas of cognitive functions (Nance, 1998).

Depression, anxiety, aggressive, impulsive and obsessive-compulsive behaviors are commonly described features and are frequently treated pharmacologically and require behavioral intervention (Holtser-Cochav et al.,

2006). However, medications used to treat these aspects of the disorder can lead to over sedation and apathy already common to HD patients. Mood disturbance is said to affect up to 40 percent of individuals with HD with poor behavioral control causing distress to not only the patient but, to family and caregivers as well. Denial of the disease and its related disabilities often requires placement in a care facility or with a full time caregiver.

1.1.6 Current Pharmacological Treatments and Management

Pharmacological interventions typically address the hyperkinetic movement disorders, but may also affect psychiatric issues or cognitive decline associated with HD. Tetrabenazine is the only US FDA-approved drug for HD and is indicated for the treatment of chorea associated with HD (Holtser-Cochav et al., 2006). Tetrabenazine reversibly inhibits the central vesicular monoamine transporter type 2 (VMAT2), selectively depleting dopamine more than noradrenaline (norepinephrine) (Holtser-Cochav et al., 2006). VMAT2 binding and monoamine depletion is reversible and last hours and is not modified by long-term treatment. The highest binding density for tetrabenazine is in the caudate nucleus, putamen and nucleus accumbens, areas known to bear the brunt of HD pathology.

1.2 Huntingtin Protein

1.2.1 Role of Wild Type Huntingtin

Human Htt is a large protein comprising 3,144 amino acids, with the polyQ domain beginning at the 18th amino acid position (Li and Li, 2004). Htt is expressed most abundantly throughout the brain but is also detected in other mammalian tissues, with no known exact function. Human Htt gene carries a normal polymorphic CAG stretch ranging from 9 to 35 repeats, where the rat and mouse genes have 8 and 7 CAG repeats, respectively (Cattaneo et al., 2001). Deletion of the mouse homolog of human Htt gene is lethal in the embryo before the brain is formed, yet heterozygote mice with one intact Htt gene develop normally, indicating Htt's importance in developmental stages of life (Bhide et al., 1996). Expression of Htt occurs early and throughout brain development, increases in parallel with the maturation of neurons in postnatal periods and is regulated developmentally in HD mouse models (Bhide et al., 1996). Htt immunoreactivity is distributed throughout the dendrites and axons of adult mouse brain neurons. Expression in the whole brain increases markedly between P7 and P15, during which it rises from approximately 40 percent to 100 percent of adult levels. During these stages neurogenesis is complete in most brain areas, axonal elaborations are being pruned, and dendrites, spines, and synapses are forming (Bhide et al., 1996). It should be noted that there is a noticeable

slower rise of Htt expression in the striatum compared with that in the cortex in postnatal mice, possibly indicating a delayed maturation of striatal neurons compared with cortical neurons (Bhide et al., 1996).

Based on the known functions of proteins that interact with Htt it is presumed to be involved in gene transcription, intracellular signaling, trafficking, endocytosis, and metabolism (Li and Li, 2004). The variety of interacting proteins with Htt suggest it may act as a scaffold involved in orchestrating sets of proteins for signaling processes and intracellular transport (Li and Li, 2004). Interactions with huntingtin-associated protein (HAP1) and huntingtin-interacting protein (HIP1) supports the theory that Htt is involved in endocytosis and intracellular trafficking pathways.

The only known structural domains in Htt are multiple HEAT(huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR1) domains that have also been identified in nuclear shuttle proteins (Cattaneo et al., 2001). The structure of Htt includes a long polyQ stretch followed by a poly-proline (polyP) domain which bears a similar characteristic of transcriptional regulatory proteins (Cattaneo et al., 2001). The nuclear transcription factors CREB (cAMP-response element binding protein) binding protein (CBP) and specificity protein 1 (SP1) have been shown to bind to Htt. Both of these proteins are important for expression of neural genes and neuronal function (Cattaneo et al., 2001).

1.2.2 Role of Mutant Huntingtin

In HD, mHtt has been shown to form insoluble aggregates in the cytoplasm and nucleus in various HD models. The formation of aggregates form only when the number of glutamine residues exceeds approximately 35, which is also the threshold for pathogenicity (Tobin and Signer, 2000). There are two theories on how these mHtt aggregates form. The polar zipper model proposes the normal protein conformation is destabilized by the presence of the expanded polyQ tract leading to abnormal protein-protein interaction and the formation of insoluble ß-pleated sheets linking ß-strands together into barrels or sheets via hydrogen bonding, forming so-called polar zipper structures (Ho et al., 2001). Whereas the alternative theory proposes transglutaminases, enzymes normally involved in crosslinking of glutamine residues in different proteins, interact with the expanded polyQ stretch of mHtt resulting in increased cross-linking between mHtt and itself or other proteins (Ho et al., 2001). The polyP domain of mHtt prevents it from misfolding and diminishes mHtt from forming self-aggregates (Kim and Kim, 2014). In vitro studies have shown that N-terminal cleavage products of mHtt are more toxic and more prone to aggregate than the full length version of mHtt (Ho et al., 2001).

One of the main suspected pathways for mHtt is its involvement in apoptosis of medium spiny neurons of the striatum. The fact that both Htt

and mHtt have cleavage sites that are specifically cleaved by caspase-3 supports the idea of some unknown pro-apoptotic pathway caused by mHtt (Ho et al., 2001). When cleaved by caspase-3 mHtt yields two smaller fragments that may further promote other caspases thus promoting further cytotoxicity and pro-apoptotic pathways (Hickey and Chesselet, 2003). Interestingly, HIP1 can induce apoptotic morphology and the interaction of HIP1 and Htt is reduced with increasing CAG repeat length, which suggests the expanded CAG repeat of mHtt allows for increased levels of pro-apoptotic HIP1 in cells.

Both Htt and mHtt have similar expression and distribution within the striatum and cerebral cortex (Kim and Kim, 2014). It is believe that mHtt is toxic to cells by affecting transcription, mitochondrial function, synaptic transmission and axonal transport by sequestering selective transcription factors and co-activators including; CBP, TATA-binding protein,p53,SP1 and TAFII-130 into aggregates (Kim and Kim, 2014). The mHtt loses its ability to retain cytoplasmic repressor element 1 transcription factor / neuron restrictive silencer factor (REST/NRSF), causing transcriptional repression of neuron restrictive silencing element (NRSE) containing genes such as brain-derived neurotrophic factor (BDNF) (Kim and Kim, 2014). Cortical BDNF is transported to the striatum and is critical to striatal neuronal activity implicated in cortico-striatal connections. Interaction of mHtt with HAP1 and p150Glued (subunit of dynactin) lead to

impared retrograde transport of BDNF (Kim and Kim, 2014). Anterograde transport is also reduced due to reduction of α-tubulin acetylation, which is important for kinesin 1 binding to microtubules (Kim and Kim, 2014).

1.3 Neurotrophic Factors in Huntington's Disease

Neurotrophic factors may increase the neuronal metabolism, cell growth and processes that can lead to the growth of new axons and the reestablishment of synaptic connections (Sari, 2011). Striatal neurons do not express BDNF mRNA, yet they still produce TrkB (a cell surface receptor for BDNF) and this trophic connection may be disturbed in HD (Kruttgen et al., 2003). Htt has been shown to upregulate BDNF where as mHtt directly inhibits transcription of BDNF due to mHtt aggregates sequestering CBP (Kruttgen et al., 2003; Sari, 2011). The sequestering of CBP might downregulate BDNF thus depriving striatal neurons of neurotrophic support because of the diminished expression and reduced anterograde transport in cortical and substantia nigra neurons in addition to reduced retrograde transport in dendrites and axons of striatal neurons (Sari, 2011).

1.4 Neurotrophic Factors D-NAP and D-SAL

Activity-dependent neuroprotective protein (ADNP) was discovered as a vasoactive intestinal peptide (VIP)-responsive glial gene (Gozes et al., 2004). The active element of ADNP was identified as an 8-amino acid peptide,

NAP (Asn-Ala-Pro-Val-Ser-Ille-Pro-Gln; single-letter code, NAPVSIPQ), and exhibits potent neuroprotective activities (Gozes et al., 2004). NAP has shown protection against the following: alzheimer's disease neurotoxin ß-amyloid, electrical blockade, oxidative stress, dopamine toxicity and decreased glutathione, excitotoxicity, N-methyl-D-aspartate, glucose deprivation, the toxic envelope protein of human immunodeficiency virus, and tumor necrosis factor-α in vitro (Gozes et al., 2004).

VIP also causes secretion of a novel protein named activity-dependent neurotrophic factor (ADNF) (Steingart et al., 2000). ADNF is structurally similar to heat-shock protein 60 (HSPS60), an intra-cellular protein that is stimulated under stress conditions (Steingart et al., 2000). A 9-amino acid peptide was identified as the active element of ADNF and was named ADNF-9 or D-SAL (single-letter code SALLRSIPA) (Gozes et al., 2008).

D-SAL and D-NAP are expected to interact with similar molecular targets based on; the high degree of similarity between D-NAP(N**A**PV**SIP**Q) and D-SAL (S**A**LLR**SIP**A) amino acid structure (identical amino acids are in bold), and that both peptides require the SIP moiety for neuroprotective activity (Holtser-Cochav et al., 2006). Both D-enantiomers of NAP and ADNF-9 maintain femtomolar-acting neuroprotective activity indicating the mechanism of action is independent of chiral recognition (Gozes and Spivak-Pohis, 2006). A study has shown both peptides may exert their action through

the activation of poly(ADP-ribose) polymerase-1 (PARP-1) (Gozes and Spivak-Pohis, 2006). Calcium-dependent kinases are involved in PARP-1 activation by NGF-like growth factors and NAP (Gozes and Spivak-Pohis, 2006). In rat pheochromocytoma cells (PC12) that exhibited differentiation induced by NGF-like growth factors and NAP, used a mechanism involving intracellular calcium – dependent poly-ADP-ribosylation, which has been suggested as a being neuroprotective by rendering DNA accessible to transcription and repair (Gozes and Spivak-Pohis, 2006).

1.5 Neuroprotective Peptide Colivelin

A 24-amino acid long peptide, Humanin (HN) and analog peptides have shown to protect neurons from a variety of neurotoxic insults (Arakawa et al., 2008). The analog AGA(C8R)-HNG-17 (single-letter code PAGASRLLLLTGEIDLP) shows activity at 10pM, which is about 10^5 – fold more active than the parent HN molecule with activity at approximately 1μM. ADNF-9 (D-SAL) is a 9-amino acid long core peptide of ADNF that is active in fM concentration, but loses the activity above 10nM for unknown reasons (Arakawa et al., 2008).

In order for HN to properly exhibit neuroprotection it is essential that it forms a dimer because ; (a) dimerization-deficient HN derivatives completely lose protective activity, (b) fusion of a dimerization tag sequence (EFLIVIKS) to the N-terminus of HN derivatives potentiates neuroprotective

activity (Chiba et al., 2007). ADNF-9 is a highly lipophilic peptide that is predicted to oligoomerize as a dimerization tag. By attaching ADNF-9 to the N-terminus of AGA-(C8R)HNG-17 it presumably enhances the dimerizing ability thus promoting the neuroprotective effect of the fused peptide in a synergistic manner (Chiba et al., 2007; Sari et al., 2009). The resulting fusion of the two peptides resulted in a new peptide named Colivelin (single-letter code; SALLRSIPAPAGASRLLLLTGEIDLP) (Sari et al., 2009). Colivelin exhibits a neuroprotective effect on various types of insults, including glutamate toxicity, possibly through its ADNF portion. Whereas HN alone does not exert any neuroprotective effect against glutamate- induced excitotoxicity suggesting Colivelin neuroprotective effect is mediated by two distinct pathways (Sari et al., 2009). The ADNF/CAMKIV pathway and the HN/STAT3 pathway appear to be activated simultaneously and independently by Colivelin (Chiba et al., 2007; Sari et al., 2009).

1.6 Rat Adrenal Pheochromocytoma Cells (PC12)

In growth medium the PC12 cells have a round or polygonal shape and tend to grow in small clumps with an apparent doubling time of approximately 92 hours (Greene and Tischler, 1976). Similarly to primary sympathetic neurons, PC12 cells are sensitive to nerve growth factor protein (NGF). In the presence of NGF PC12 produce fine fibers reaching 500-1,000µm that branch profusely, have numerous varicosities , and even form

fascicles (Greene and Tischler, 1976). This effect caused by NGF is reversible in that removal of NGF does not appear to affect the integrity of the cell bodies despite it undergoing a degeneration process (Greene and Tischler, 1976). The ultrastructure of PC12 contain round, ovoid or somewhat irregular dense core granules in growth media with or without NGF, however in the presence of NGF small round vesicles of approximately 20-70nm in diameter can be seen (Greene and Tischler, 1976). These vesicles frequently aggregate towards the ends of extended fibers (Greene and Tischler, 1976). PC12 cells possess the pluripotency of primitive progenitor which can differentiate along the lines of either chromaffin cells or sympathetic neurons, with NGF promoting their differentiation in a neuronal direction (Greene and Tischler, 1976).

1.7 Aims and Objectives

The aim of this study was to evaluate the effects of three related neurotrophic peptides in a cell-based assay for HD. The assay measures the ability of compounds to protect cultured PC12 cells from death caused by an expanded polyQ form of mHtt exon 1. D-NAP, D-SAL, and Colivelin have all shown neuroprotective activity in various other neurodegenerative diseases but have yet to be extensively evaluated for neuroprotective activity in HD. All three peptides were tested at six different concentrations (fM-nM) by

either simultaneous exposure to the HD inducer and neurotrophic peptide or twenty-four hours pre-incubation with peptide before adding the HD inducer.

Chapter 2

Materials and Methods

2.1 Plasmids

The plasmids used for transfecting mammalian cells contained a synthetic DNA insert encoding exon 1 of human Htt^{Q103} fused to EGFP. These inserts were originally gifts of Alex Kazantsev and David Housman (MIT), and encode the entire exon 1, including the proline-rich segment, and containing 103 mixed CAG/CAA repeats, fused to a C-terminal EGFP tag in pcDNA3.1/myc-HIS (Invitrogen). This insert, previously called 104Q/EGFP (Kazantsev et al., 1999), was subcloned into pBWN, a gift from Steve Suhr and Fred Gage (Salk Institute). pBWN is an ecdysone-responsive expression vector containing a neo^r gene to allow for G418 selection of stable clones (Suhr et al., 1998). Transgene expression was induced by the addition of 1μM tebufenozide (TEB), an ecdysone analog. Tebufenozide does not have any known deleterious effects on mammalian cells(Aiken et al., 2004).

2.2 Cell Culture

2.2.1 PC12 Growth Conditions

PC12 cells with the mHtt^{Q103} (referred to as PC12^{Q103}) plasmid were purchased from Dr. Erik Schweitzer lab in 2011. Stock PC12^{Q103} were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 25mM HEPES (Mediatech #15-018-CV), +5% supplemented calf serum (Hyclone), +5% heat-inactivated horse serum (Hyclone), 2mM L-glutamine, penicillin and streptomycin, at 37˚C in 5% CO_2.

2.2.2 PC12 Stock Maintenance

2.2.2.1 Aseptic Techniques

When working with the PC12^{Q103} cells aseptic techniques were implemented in a Thermo Scientific cell culture hood 1300 series A2. All consumables were either bought previously sterilized or manually sterilized via; exposure to UV light, autoclave, or filter sterilization.

2.2.2.2 Frozen PC12 Stock

Stock PC12^{Q103} were frozen in pre-chilled growth medium containing 10% dimethyl sulfoxide (DMSO) and placed in a freezing container,

Nalgene® Mr. Frosty (C1562 SIGMA) filled with isopropyl alcohol. The $PC12^{Q103}$ were allowed to freeze overnight in a -80˚C freezer before being placed into liquid nitrogen for storage.

2.2.2.3 Thawing PC12 Stock

$PC12^{Q103}$ growth medium was pre-warmed to 37˚C in a Thermo Scientfic Microprocessor Controlled 280 Series water bath. Vials containing $PC12^{Q103}$ cells were allowed to thaw for 1 to 3 minutes in the 37˚C water bath. Once thawed the vial containing cells was transferred to a 15ml centrifuge tube containing 5ml of growth medium. The 15ml centrifuge tube containing cells was then placed in an Eppendorf Centrifuge 5810 at 2,000 rpm for 5 minutes. The supernatant was removed and the remaining $PC12^{Q103}$ pellet was re-suspended in growth medium. The $PC12^{Q103}$ cell suspension was then counted (method described elsewhere) and plated at a density of $2.2x10^6$ in 10ml growth medium within a 100mm cell culture dish. Dishes were labeled with: date of thawing, passage number, cell line, and initials of individual.

2.2.2.4 Determining Cell Concentrations

Equal volumes of cell suspension and 0.4% trypan blue in CA-MG HBS were incubated at room temperature for 5 min. A total of 10µl of trypan

blue/cell suspension was loaded into the well of a hemocytometer by capillary action. Cells were viewed using a Leica DM 1000 microscope at a magnification of x10 and cells excluding the dye were counted. The concentration of cells was determined by taking the average live cell count of the large four corner squares of the hemocytometer and multiplying by the dilution factor and by the volume of liquid on the hemocytometer ($1x10^4$ml) to obtain the number of cells per ml.

2.2.3 Tebufenozide Time Course

$PC12^{Q103}$Cells and $PC12^{Q25}$ (as a positive control) at a concentration of $0.3x10^6$ cells were plated in separate 35mm cell culture dish in a total of 2ml growth medium and allowed to grow to approximately 80% confluency. Growth medium was then supplemented with 1µM tebufenozide (TEB) and replaced the 2ml of growth medium without TEB. Cell viability was then assessed at 0, 6, 12, 24, 48, and 72 hours after adding growth medium containing 1µM of the $mHtt^{Q103}$ inducer TEB. The percent of viable cells was determined by trypan blue exclusion and cell concentrations by hemocytometer.

2.2.4 LDH Assay for Cell Viability

2.2.4.1 Simultaneous Exposure to 1μM TEB and Neurotrophic Peptide

Each well of a 96-well plate was seeded with approximately $2x10^4$ $PC12^{Q103}$ cells in 200μl of growth media and allowed to attach for 24 hours. The following day growth medium was replaced with one of the following treatment types: growth medium (untreated control), growth medium + 1μTEB (negative control), growth medium + 1μTEB + 50μM BOC-D-FMK (positive control), or growth medium containing one of the neurotrophic peptides (D-SAL, D-NAP, Colivelin) at a concentration of 10fM,100fM,1pM,10pM,100pM,1nM + 1μTEB, respectively. 50μM BOC-D-FMK was used as positive control as described in (Aiken et al., 2004). Blank wells were prepared by treating wells containing medium but no cells. Each well type was prepared in triplicate.

After 48 hours a x2 LDH reaction mix consisting of 1x PBS (pH7.4), 0.01M NAD^+ (Sigma), and 0.2M sodium lactate (Sigma) was freshly made before every assay. From plates containing cells, 100μl of the media was removed and placed into a separate 96-well plate, 100μl of x2 LDH reaction mix was then added to the 100μl of media immediately before measurement of change in absorbance was recorded by a Biotek synergy ht plate reader.

LDH activity was measured by the change in absorbance at 340nm over a period of 5 min and calculated the slope of absorbance over time.

2.2.4.2 24 Hour Pre-incubation With Neurotrophic Factor

Each well of a 96-well plate was seeded with approximately $2x10^4$ $PC12^{Q103}$ cells in 200µl of growth media containing one of the neurotrophic peptides (D-SAL, D-NAP, Colivelin) at a concentration of 10fM,100fM,1pM,10pM,100pM,1nM, respectively and allowed to settle for 24 hours. The growth media was then replaced and LDH activity was measured 48 hours after addition of 1µTEB as previously stated.

2.3 Statistical Analysis

The change in absorbance over time was calculated for each individual well and the average change in absorbance of blank wells was subtracted to remove background absorbance. In order to account for variance between a total of three experiments the change in absorbance was normalized by dividing the change in absorbance of each individual well by the average absorbance reading of the untreated controls and multiplied by 100 to obtain a percent in comparison to the untreated control group. The means of the percent of untreated control groups from all three experiments were calculated and shown graphically. A one-way ANOVA was employed to determine differences between the three control groups and treatment groups using GraphPad Prism version 5 statistical software. Statistical significance

was set at $p < 0.05$ and determined by using LSD post hoc analysis for both exposure methods tested.

Chapter 3

Results

3.1 Tebufenozide Time Course

Inducing expression of Htt^{Q103} by adding tebufenozide to the culture media caused a progressive and rapid cell death determined by trypan blue exclusion of cells at the time intervals of 0, 6, 12, 24, 48, and 72 hours post (data not shown). Figure 1 shows $PC12^{Q103}$ in growth media exhibit above ninety percent viability whereas $PC12^{Q103}$ with growth media containing tebufenozide exhibited less than fifty percent viability after 48 hours. Addition of tebufenozide to the culture media of PC12 cells expressing Htt^{Q25} ($PC12^{Q25)}$ had little to no effect on cell survivability as seen in Figure 1.

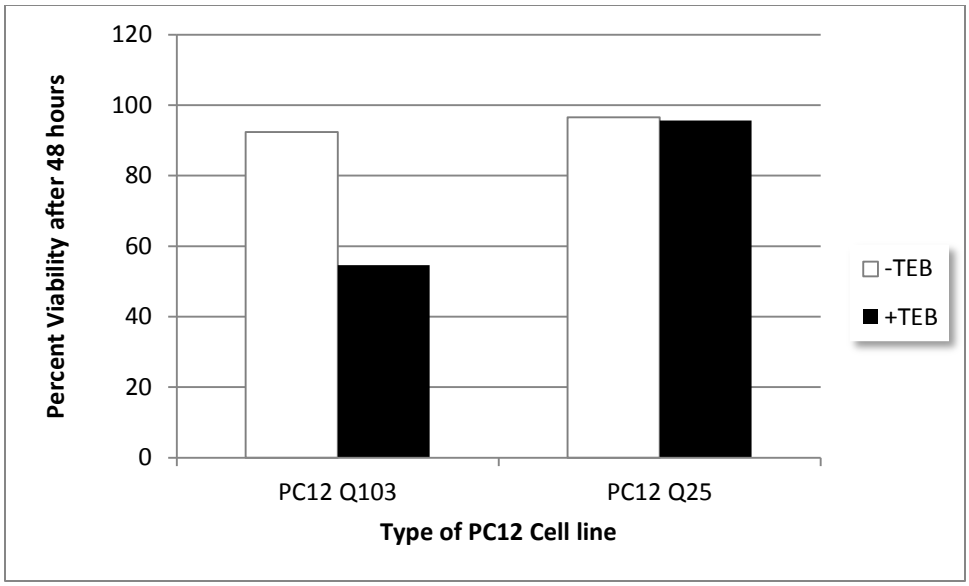

Figure 1. Percent viability (live cells/total cell count) of PC12 Q103 or Q25 cells in the presence or absence of tebufenozide after 48 hours

Activation of the Htt^{Q103} construct (green staining) was seen expressed diffusely throughout the cytoplasm of $PC12^{Q103}$ cells as well as some aggregate formation despite the absence of the Htt^{Q103} inducer TEB in culture media as seen in Figure 2A. Whereas $PC12^{Q103}$ cells exposed to culture media with 1μTEB for forty-eight hours resulted in aggregate formation within the nucleus (blue staining) of $PC12^{Q103}$ cells. Figure 2C shows $PC12^{Q25}$ cells with activated Htt^{Q25} construct after forty-eight hours of incubation in culture media without the presence of TEB. After forty-eight hours of incubation with the presence of 1μTEB a larger portion of $PC12^{Q25}$ cells show diffuse cytoplasm expression of the Htt^{Q25} construct with no observed aggregate formations as seen in Figure 2D.

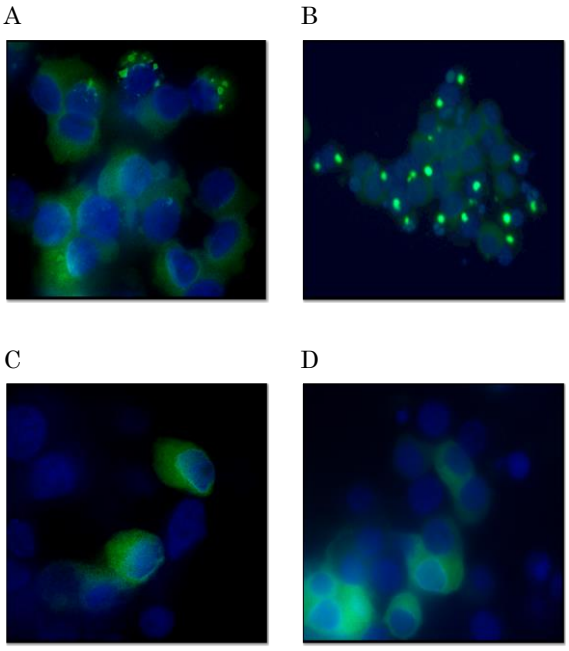

Figure 2. A.) $PC12^{Q103}$ cells in growth medium, B.) $PC12^{Q103}$ cells in growth medium containing 1μTEB, C.) $PC12^{Q25}$ cells in growth medium, D.) $PC12^{Q25}$ cells in growth medium containing 1μTEB. All images were collected after 48 hours of exposure to the designated growth medium. Green florescence indicates activation of the Htt construct and blue florescence is the result of DAPI stained nuclei of the PC12 cells.

3.3 LDH Assay of Simultaneous Exposure to 1μM TEB and Neurotrophic Peptide

One-way ANOVA of three separate experiments for simultaneous exposure to 1μM TEB and a single neurotrophic peptide at one of six concentrations revealed a significant difference between the treatment types at a $p<0.05$ (D-NAP: [$F(8,62) = 2.432$, $p = 0.023$], D-SAL: [$F(8,63) = 3.968$, $p = 0.001$], Colivelin: [$F(8,63) = 2.646$, $p = 0.014$]). LSD Post-Hoc test comparing the negative control to a treatment with neurotrophic peptide indicated a significant difference in LDH activity for D-SAL (Figure 4) at concentrations

100fM,10pM,100pM,1nM and Colivelin (Figure 5) at concentrations 100fM,and 100pM. Simultaneous treatment with D-NAP (Figure 3) did not show significant difference at any of the tested concentrations when comparing it to the negative control.

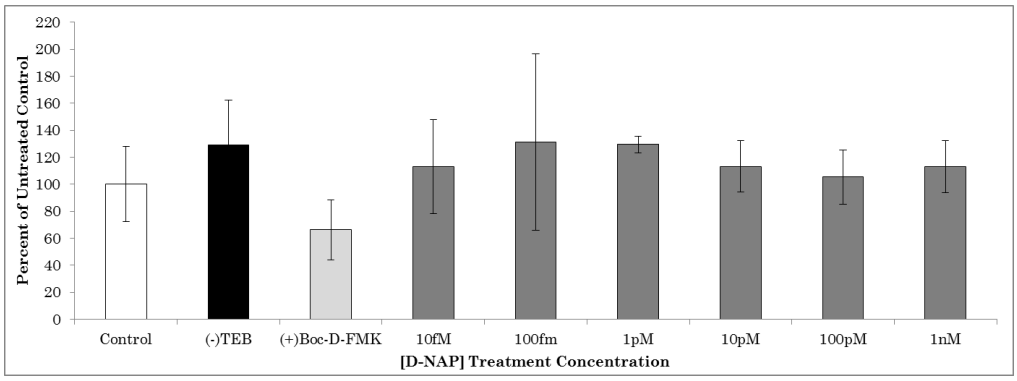

Figure 3. Means from the percent of untreated control ± SD represent LDH activity after 48 hours. Untreated control (white bar), Negative control (TEB, solid black bar), Positive control (Boc-D-FMK, light grey bar), D-NAP treatment (dark grey bar)

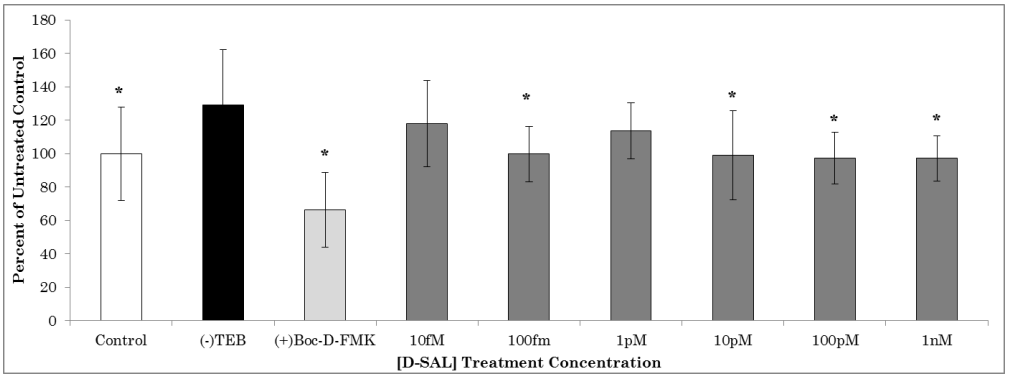

Figure 4. Means from the percent of untreated control ± SD represent LDH activity after 48 hours. Untreated control (white bar), Negative control (TEB, solid black bar), Positive control (Boc-D-FMK, light grey bar), D-SAL treatment (dark grey bar). Statistical significance determined by LSD Post-Hock is represented as an asterisk (*****) when compared to negative control: Untreated control ($p = 0.011$), Positive control ($p = 0.00$), 100fM ($p = 0.010$), 10pM ($p = 0.009$), 100pM ($p = 0.006$), 1nM ($p = 0.013$).

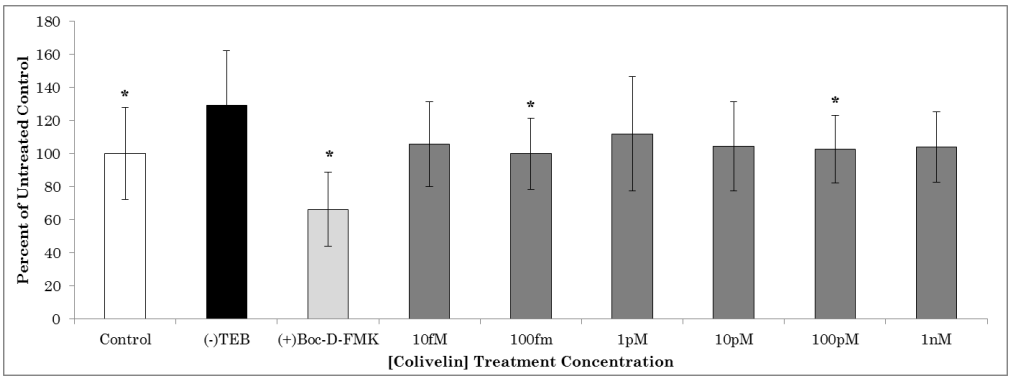

Figure 5. Means from the percent of untreated control ± SD represent LDH activity after 48 hours. Untreated control (white bar), Negative control (TEB, solid black bar), Positive control (Boc-D-FMK, light grey bar), Colivelin treatment (dark grey bar). Statistical significance determined by LSD Post-Hock is represented as an asterisk (*****) when compared to negative control: Untreated control ($p = 0.019$), Positive control ($p = 0.00$), 100fM ($p = 0.022$), 100pM ($p = 0.037$).

3.4 LDH assay of 24 hour pre-incubation with neurotrophic peptide

One-way ANOVA of three separate experiments for 24 hour pre-incubation with a single neurotrophic peptide at one of six concentrations revealed a significant difference between the treatment types at a $p<0.05$ (D-NAP: [$F(8,67) = 7.590$, $p = 0.000$], D-SAL: [$F(8,66) = 3.857$, $p = 0.001$], Colivelin: [$F(8,65) = 7.440$, $p = 0.000$]). LSD Post-Hoc test comparing the negative control to a treatment with neurotrophic peptide indicated a significant difference in LDH activity for: D-NAP (Figure 6) at concentrations 100fM, 10pM, and 1nM, D-SAL (Figure 7) at a concentration of 10fM, and Colivelin (Figure 8) at concentrations 10fM, 100fM , and 10pM.

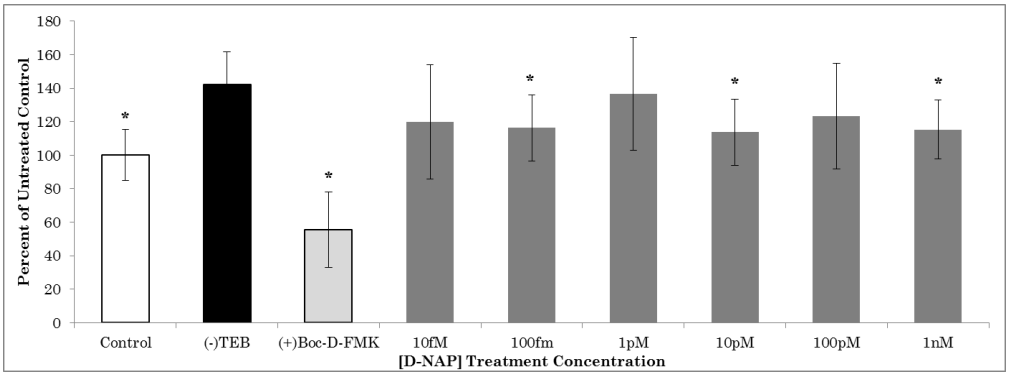

Figure 6 Means from the percent of untreated control ± SD represent LDH activity after 48 hours. Untreated control (white bar), Negative control (TEB, solid black bar), Positive control (Boc-D-FMK, light grey bar), D-NAP treatment (dark grey bar). Statistical significance determined by LSD Post-Hock is represented as an asterisk (*****) when compared to negative control: Untreated control (p = 0.001), Positive control (p = 0.00), 100fM (p = 0.032), 10pM (p = 0.019), 1nM (p = 0.031).

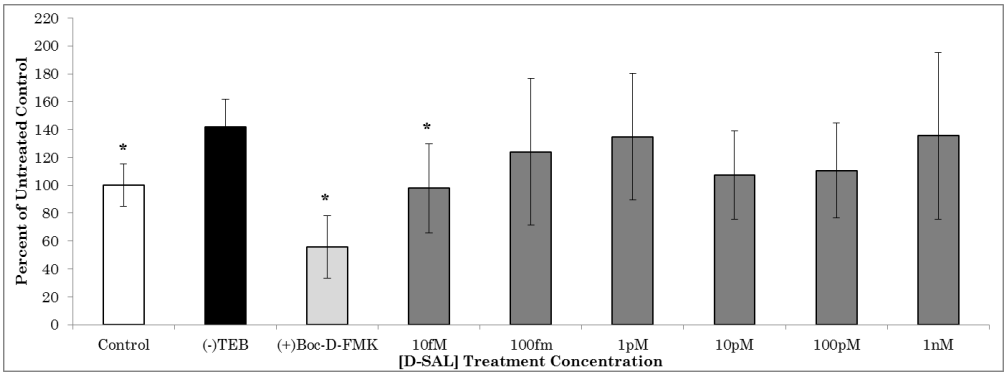

Figure 7 Means from the percent of untreated control ± SD represent LDH activity after 48 hours. Untreated control (white bar), Negative control (TEB, solid black bar), Positive control (Boc-D-FMK, light grey bar), D-SAL treatment (dark grey bar). Statistical significance determined by LSD Post-Hock is represented as an asterisk (*****) when compared to negative control: Untreated control (p = 0.023), Positive control (p = 0.00), 10fM (p = 0.014).

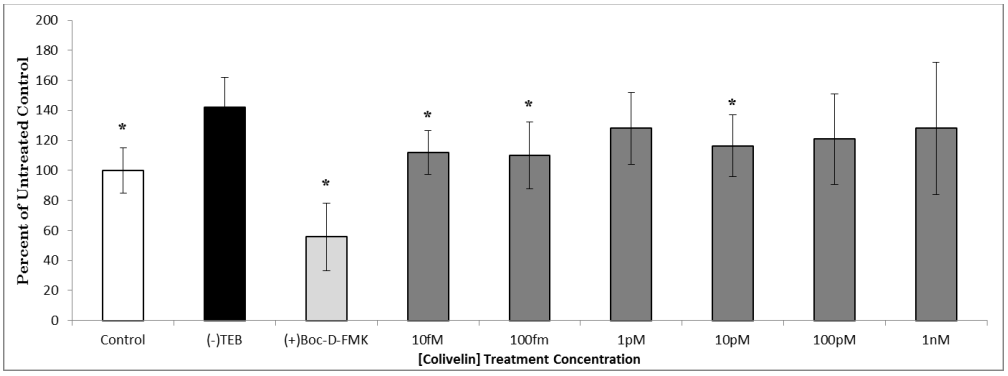

Figure 8 Means from the percent of untreated control ± SD represent LDH activity after 48 hours. Untreated control (white bar), Negative control (TEB, solid black bar), Positive control (Boc-D-FMK, light grey bar), Colivelin treatment (dark grey bar). Statistical significance determined by LSD Post-Hock is represented as an asterisk (*****) when compared to negative control: Untreated control (p = 0.019), Positive control (p = 0.00), 10fM (p = 0.012), 100fM (p = 0.008), 10pM (p = 0.031).

Chapter 4

Discussion

4.1 Conclusions

4.1.1 Simultaneous Exposure to TEB & Neurotrophic Peptide

The significant difference of LDH activity exhibited by activated $PC12^{Q103}$ treated with D-SAL at concentrations 100fM, 10pM, 100pM and 1nM appears to delay or inhibit cell death caused by the activated Htt^{Q103} construct. Colivelin exhibited significant difference of LDH activity at treatment concentrations of 100fM and 100pM. The difference in treatment concentrations between D-SAL and Colivelin may be associated with the overall size of each compound, thus altering the ability of the compound to cross the plasma membrane of the $PC12^{Q103}$ cells. Another possibility may be associated with the fusion of HN to D-SAL to form Colivelin alters the

mechanism of action exerted by D-SAL rendering it less effective. The lack of significant difference in LDH activity in $PC12^{Q103}$ cells treated with D-NAP is interesting in that the structural similarities of D-NAP and D-SAL would point towards similar molecular targets. Further studies into the mechanism of action of neurotrophic peptides and the interactions with mHtt may provide insight into potential therapies to either prevent or slow the progressive neurodegenerative properties exerted by mHtt.

4.1.2 Twenty four hour pre-incubation with neurotrophic peptide

Pre-treatment with neurotrophic peptides was tested on the basis that the $PC12^{Q103}$ may require a period of time to allow therapeutic levels to be reached before activation of the Htt^{Q103} construct that exerts rapid cell death. The fact that D-NAP showed significant difference in LDH activity at concentrations of 100fM, 10pM, and 1nM with a twenty four hour pre-incubation period but, not when simultaneously administered supports this theory. Colivelin reduced LDH activity at concentrations of 10fM, 100fM and 10pM whereas D-SAL was only effective at lowering LDH activity at the concentration of 10fM. The difference between these two compounds effective concentrations may be due to the rapid break down of D-SAL in culture whereas Colivelin is able to remain stable and provide a longer duration of action.

4.2 Limitations of the Study

Establishing and maintaining the PC12^{Q103} cells from the frozen state was met with numerous complications. A factor that may have played a role in the difficulty of working with the PC12^{Q103} is what appeared to be an unidentifiable contamination within the culture dish. The contamination could only be seen as a small black dot significantly smaller than the PC12^{Q103} cells and exhibited Brownian movement. The microscopic particle did not appear to affect the PC12^{Q103} cells' viability but its presence in culture should be noted. Cultures without the addition of TEB did exhibit random clusters of cells that showed Htt^{Q103} aggregate formation when viewed under fluorescence microscopy for GFP. It is possible that a contaminant with similar structural properties as TEB was present in the PC12^{Q103} culture medium and caused the Htt^{Q103} construct to be expressed. The expression of the Htt^{Q103} in untreated PC12^{Q103} would account for the similarities of LDH activity between the untreated control group and the group exposed to TEB (negative control).

The peptides used in this study were previously purchased and used in studies of the early 2000's. It is possible that upon breaking the original seal and prolonged storage conditions degraded the peptide thus lowering its original potency making it difficult to reach therapeutic levels within the culture. It is also possible that the necessary receptors required for the

neurotrophic peptides to exert their mechanisms of action are not present in the PC12^{Q103} undifferentiated state.

The wide range of LDH activity variability between experiments may be associated with uneven distribution of cells when transferring them to a 96 well plate. The possibility of a leaky Htt^{Q103} would also alter the total number of cells that survived the transfer process.

4.3 Future Aspects of the Study

Implementing a new protocol involving NGF to differentiate the PC12^{Q103} into a more sympathetic neuron like state may prove to be a more accurate representation of HD. Also, establishing a new plasmid construct involving fewer CAG repeats may also provide a more accurate cellular model of HD that does not exhibit such rapid cell death not typically seen in HD.

In theory, neurotrophic factors should assist in prolonging cell survivability and potentially restore some function in striatal cells. The fact that pre-incubation of PC12^{Q103} with D-NAP, D-SAL and Colivelin showed a decrease in LDH activity may support the theory that medium spiny striatal cells undergo cell death due to depleted neurotrophic factors. Further evaluation of D-NAP, D-SAL and Colivelin should be conducted with newly synthesized peptides and be evaluated using a more reliable cell viability assay or in a different model of HD.

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