MT1 RECEPTOR MEDIATED NEUROPROTECTION IN R6/2 MOUSE MODEL OF HUNTINGTON'S DISEASE

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ABSTRACT

Huntington's Disease (HD) is a fatal, autosomal dominant, neurodegenerative disorder caused by a CAG repeat expansion in the huntingtin gene. The disease is characterized by chorea, as well as psychiatric and cognitive symptoms. At present, no treatment able to modify the disease progression is available. HD is characterized by the death of medium striatal spiny neurons within the brain. Melatonin is a hormone which acts as a neuroprotectant in a variety of neurodegenerative diseases. While most endogenous melatonin is produced by the pineal gland and circulated in the bloodstream, melatonin is also present in neurons. Here I use a novel method of detecting site specific melatonin synthesis to show melatonin is synthesized in neuronal mitochondria, where melatonin receptors are also localized. Previous research shows the melatonin receptor MT1 to be the mechanism of melatonin's neuroprotection in cell and mouse models of HD. Here I measure the expression of MT1 in the R6/2 mouse model of HD to compare with literature reported decrease in expression, and test the effects of the R6/2 phenotype on overexpression of MT1 in a transgenic MT1 mouse model (NSE-MT1). Further, I use the cross of the NSE-MT1 mouse model and R6/2 to assess the effects of overexpression of MT1 in increasing melatonin's neuroprotective properties to ameliorate disease phenotypes including

caspase activation, neuronal density, rotarod behavioral testing, and survival. Together, these data show overexpression of MT1 produces small increases in melatonin's neuroprotective properties with regard to caspase activation, but this protective effect does not cause corresponding increases in behavioral deficits or survival. Neuroprotection by melatonin is partially mediated by the MT1-receptor in an HD mouse model, but may additionally function by alternate pathways or be modulated by regulatory mechanisms. Understanding melatonin's role as a neuroprotectant can help with the development of melatonergic therapeutics to treat HD and other neurodegenerative diseases to significantly impact public health outcomes.

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1.0 INTRODUCTION

1.1 HUNTINGTON'S DISEASE

George Huntington first described a hereditary disease with a defining symptom of chorea in 1872 when he observed a rare disease that occurred among select families of Long Island[1]. Although Huntington's disease (HD) had a distinct genetic signature, it was over a century later that a causative gene, originally called IT15 for its interesting transcript, was mapped to 4p16.3[2]. Shortly after, a single gene, huntingtin (*HTT*), was identified, with the disease causing element found to be an expanded trinucleotide repeat of CAG in the first exon of the gene[3]. *HTT* is 180kb, with 67 exons ranging in size from 48-341 base pairs in length[4].

HD occurs in approximately 5.7 per 100,000 individuals in North America, with an estimated 30,000 individuals with manifest HD in the United States. The disease prevalence varies greatly by population, attributed to different haplotypes of average CAG tract size. HD prevalence is 10 times greater in European populations compared to Asian and African populations[5]. The disease is passed with an autosomal dominant pattern of inheritance.

The CAG expansion in exon 1 and the correlations between repeat length and HD manifestation are extensively studied. Individuals with fewer than 27 repeats have no disease risk. An intermediate range of 27-35 CAG repeats is not disease causing in individuals, but is considered a higher risk for transmitting a pathogenic repeat length to offspring[6]. Individuals with 36 or more repeats are at risk for developing HD within their lifetime. A very strong negative correlation exists between CAG repeat length and age of disease onset[7-10]. While a longer repeat length correlates with an earlier age of onset, the type of symptoms and order of their manifestation does

not seem to be determined by repeat length[9]. However, while individuals may appear similar in their symptoms and disease onset, those with longer repeats tend to have a faster disease progression[8].

While the correlation is strong, repeat length does not account for all variability in age of onset. In an extensive study among Venezuelan HD populations, repeat length was determined to account for about 70% of variability in age of onset[11], although other work estimates it closer to 50%[9]. Among the Venezuelan population, the remaining variability was estimated to be 40% due to other genes and 60% due to environmental factors. Follow-up linkage analysis mapped specific loci for other genes, which may modify *HTT*, but individual genes have not been identified[12].

HD is subject to a strong anticipation effect. The CAG repeats in the *HTT* gene are often unstable, and the repeat length can increase from parent to child. It is via this anticipation that intermediate repeat length individuals can pass a pathogenic repeat length to their offspring. This effect is more likely when the disease-causing allele is passed paternally. One theory for this is that the frequent production of sperm allows for a greater chance of mitotic instability[13]. Even within individuals, there can be instability and variation in CAG repeat length. Some instability occurs within somatic tissues, including different neuronal cell types with a high amount of variability in striatal cells[14]. However, because the disease causes neurodegeneration, it is not clear whether CAG variability in neuronal cells is due to mutant *HTT* causing selective cell death or if the neurodegeneration causes mitotic instability.

HD symptoms in adults typically present around 35-45 years of age, although about 3-10% of cases are juvenile, occurring in individuals under 21 years of age, a subset with very high CAG repeat length[15,16]. The hallmark symptom of the disease is chorea; brief, non-repetitive, non-rhythmic jerking movements. However, in a prodromal stage before chorea and other physical

symptoms manifest, individuals show a shift in mood and cognitive difficulties[17]. Patients often under-notice or are unaware of these early symptoms, a characteristic which may itself be caused by the disease[18]. Cognitive difficulties include problems with memory, visuospatial cognition, and ability to manipulate learned information. In juvenile HD, cognitive symptoms can be seen via academic ability, difficulties reading, and regression of language skills[15]. As many as half of all HD patients present with psychiatric symptoms prior to motor symptoms, although the type, severity, and onset of these psychiatric symptoms are not correlated with CAG repeat length[19]. Depression is common, and the suicide rate among HD patients is up to 10 times higher than that of the general population[17]; an estimated 30% of patients attempt suicide at least once after their diagnosis[20]. Patients also frequently report problems with sleep patterns, both in adult and juvenile variants of the disease. As the disease progresses, the more distinct physical symptoms manifest. Chorea is a common marker of disease onset, although it is not an indicator of disease severity. Dystonia, which causes involuntary muscle movements, is associated with earlier onset[21]. Another symptom, motor impersistence, is characterized by an inability to sustain voluntary muscle movements; because its presence is not correlated with presence of chorea, motor impersistence may be a particularly consistent marker of disease progression. HD patients show a clear decrease in body mass index (BMI), which is progressive over the course of the disease, and the severity of weight loss is correlated with CAG repeat length[22-24]. Patients exhibit no endocrine or gastrointestinal abnormalities, and the change in BMI appears to be due to a hypermetabolic state, although the exact mechanism is unclear[25]. In juvenile HD, seizures occur in 30-50% of cases[15]. While many symptoms are common within the disease, they do not establish a clear cause of death in HD patients. Leading causes of death for HD are pneumonia, cardiovascular disease, wasting, and suicide[26].

HD has clear neuropathology. Patients have a dramatic decrease in brain weight, which can be as much as 30% smaller than a non-HD brain at autopsy. This is due to atrophy and neuronal death, beginning in the caudate nucleus and putamen. By late stage disease, up to 95% of neurons may be lost in the caudate nucleus[27]. As the disease progresses, whole brain atrophy occurs, and this combination of neuronal death and atrophy can occur before clinical symptoms begin to manifest[28]. Neuronal death begins selectively, primarily affecting medium spiny neurons in the caudate, with the earliest loss seen specifically in GABA/enkephalin neurons. As the disease progresses, loss of cortical neurons is likely the cause of cognitive and behavioral symptoms, as well as affecting motor pathways resulting in chorea[29].

Recent research in HD shows the strong role apoptosis plays in neurodegeneration, and indicates this may be a mechanism for the cellular damage seen in the disease. Programmed cell death is seen throughout neurodegenerative diseases including Alzheimer's and ALS, as well as in stroke.

In programmed cell death, a biochemical cascade is set off which includes the activation of caspases and results in fragmentation of the nucleus. The cascade is a complex interplay of molecular components, and while the process is a natural function, a change in any one step can result in undesired cell death.

Caspases, short for cysteine-dependent aspartate specific proteases, are primary factors in apoptosis. Since the discovery of caspase 1 (or interleukin-1β-converting enzyme), a total of 11 caspases are identified in humans. These small molecules begin as procaspases, molecules with a recruitment domain on the N-terminal. Variety in the recruitment domains inform where along the cell death cascade each caspase functions: upstream or downstream, initiating or executing. The N-terminal domain of most upstream molecules (procaspases 1, 2, 4, 5, 9, 11, 12, 13) is a

caspase-recruiting domain (CARD). There are also two caspases (8 and 10) which have a death-effector domain (DED) at the N-terminus. These upstream caspases function to initiate the process of apoptosis. The other caspases (3, 6, 7, 14) have a short prodomain[30]. These caspases function in actual executing of the cell by both destroying cellular structures and degrading DNA[16].

Apoptosis can occur through intrinsic mitochondrial mediated pathways or extrinsic death receptor pathways. While apoptosis has traditionally been described more broadly, more recent understanding has noted that this type of cell death is defined by the activation and involvement of caspases[31]. Initial signaling to induce this caspase-mediated cell death pathway can come from increased levels of free radicals or intracellular calcium, or from pro-apoptotic signals from proteins of the BCL2 family. Proteins within the BCL2 family can act as inhibitors or promoters of apoptosis[32,33]. For example, upstream of the mitochondria, caspase 1 cleaves Bid, a protein of the Bcl-2 family [34]. After this cleavage, the truncated Bid protein (tBid) translocates to the mitochondrial membrane. There, it interacts with BCL2 family proteins Bcl-X_L and Bax, resulting in a release of cytochrome c from the mitochondria[35].

These signals lead to the release of apoptotic factors from the mitochondria, following both caspase dependent and caspase independent pathways. Critically to the caspase mediated cell death, cytochrome c is released from the mitochondria. Cytochrome c then binds Apaf-1 and pro-caspase 9 to form the apoptosome and activate caspase 9[36]. Activated caspase 9 then cleaves and activates caspase 3, which proceeds to effect cell death.

Cell death is an important regulatory element in biological systems. Disrupting the caspase mediated cell death pathway can have significant negative effects in a healthy organism. For example, caspase 9 knockout mice show overgrowth and malformation of brain tissue, due to a

lack of apoptosis during development[37]. Conversely, in many neurodegenerative diseases, caspase activity is increased and apoptosis function is altered[38].

Notably, in HD, several caspases are activated. In both mice and humans, caspase 1 showed increased activity beginning early in the disease progression, while inhibition of caspase 1 slows the disease progression in the R6/2 mouse[39]. Similarly, caspase 3 activation is increased in both mouse models and human patients, and inhibition results in extended survival of the R6/2 model[40]. Additionally, caspase 9 activation and cytochrome c release are also increased in HD human and HD mouse model brains[41]. The increased activity of caspase 3 and 9 occurs later in the disease progression, whereas increased caspase 1 activity is seen earlier[39,41]. While the changes in caspase activity illustrate a key mechanism of HD pathology, they also provide an important mechanism for treatment as the restoration of their appropriate activity results in clear improvement to the disease phenotype.

1.1.1 HD Mouse Models

Due to its clear genetic cause, HD is particularly well suited for animal modeling. There are several methods by which mouse models of HD are generated. Models use either an insertion of human *HTT* or alter the mouse homolog, the *Hdh* gene[42]. Knock-in of CAG repeats into the mouse *Hdh* gene include models such as HdhQ111, which uses the endogenous *Hdh* promoter. These knock-in models are created by either expanded repeats in the mouse gene or, as is the case in the zQ175 model, with a chimeric exon 1 combining both human and mouse regions[43]. Insertion of the human *HTT* is done with either a fragment or full length model[44]. Full length models have the entirety of the human *HTT* gene inserted into the mouse genome using yeast or bacterial artificial chromosomes. Common full length models include YAC128 and BacHD[44]. Because the

pathogenic polygluatmine repeat in humans is in the first exon of the *HTT* gene, some models insert only the first exon of human *HTT*. Common fragment models include R6/2 and N171-82Q. Promoters vary among models[42]. While full length models are genetically more similar to human HD, the fragment models tend to produce a more robust observable phenotype[45].

Because of the variation in the gene, polyglutamine tract size, and promoter variations, it is difficult to compare studies between mouse models[46]. Additionally, characterizing the modeled aspects of motor, cognitive, and psychiatric symptoms can be difficult in mice due to the way these overlap in their manifestation[44]. Dysfunction in one aspect may be traced to several possible affected neuronal pathways. However, despite these difficulties, HD model mice provide critical insight into the disease pathology, particularly useful for measuring therapeutic outcomes.

One of the most common and best characterized of the HD mouse models is the R6/2 mouse. This first mouse model of HD was developed by Mangiarini, et al., in 1996 as one of several R6 mice, each with a different number of CAG repeats in the exon 1 insert. All R6 mice use the human *HTT* promoter. The R6 mice were developed on a hybrid background of F1 offspring of CBA and C57/BL6 mice (B6CBAF1). While attempts were made to maintain the line only mating with C57/BL6, poor litter sizes required that the R6 lines be bred on a background with more robust breeding, the B6CBA line. Of the original lines, R6/1 and R6/2 continued to be used for follow-up experimentation, with R6/2 being the most common.

R6/2 mice average around 144 CAG repeats[47]. This polyglutamine track is considerable larger than even most juvenile HD patients and results in a very severe and rapid phenotype. R6/2 mice present with a progressive phenotype with onset around 7-8 weeks of age, which rapidly becomes more severe and ends in death around 13-14 weeks of age[47].

Similar to patterns of symptoms in human disease, the mice exhibit motor symptoms including chorea-like movements, mild ataxia, resting tremor, and involuntary stereotypic movements such as stroking of the face and kicking of hindlimbs. In tests of motor performance and grip strength, the R6/2 mice show progressive decline with age[45]. The mice exhibit normal weight gain from weaning until disease onset when they plateau and then begin to lose weight. This weight loss is consistent in both male and female R6/2, although males exhibit more severe loss[45]. Despite continuing to eat normally, R6/2 mice lose both body weight and muscle mass, similar to what is seen in human HD patients[47]. While approximately 25% of R6/2 mice develop diabetes mellitus, this does not appear to affect survival, weight loss, or motor test performance[48].

As in juvenile HD, R6/2 mice occasionally have seizures, although these are not well characterized and the cause is unknown. Rarely, mice die as a result of seizure (less than 2% of deaths)[45]. No comprehensive work currently exists documenting cause of death in R6/2 mice[46]. The second most common cause of death in HD patients is cardiovascular defects[26,49], and R6/2 mice show cardiac dysfunction as well. Reduced cardiac output seen at 8 weeks and by 12 weeks, heart weight is decreased[49]. This may be a significant contributing factor to R6/2 death, and is currently the only potential cause that has been investigated. While cause of death is not well understood, survival is consistently used as a reliable indicator of neuroprotection in therapeutic studies[45].

R6/2 mice model several key pathological features which are believed to individually or collectively influence HD in humans, including protein aggregates, mitochondrial dysfunction, synaptic dysfunction, and cell death and autophagy[46]. The brains of R6/2 mice are significantly smaller than those of wild type littermates, with approximately 19% reduction seen by late stage disease. At both 60 and 90 days of age, significant loss is seen specifically in striatal neurons; this

loss is believed to account for a large portion of overall reduction in brain mass[45]. R6/2 mice also are one of the few HD models which have intranuclear inclusions; these protein aggregates are most prominent in striatal neurons with 98% of these cells affected by 12 weeks[46].

1.2 MELATONIN & SIGNALING

1.2.1 Melatonin

N-acetyl-5-methoxytryptamine, better known as melatonin, is a hormone found in mammals synthesized from the amino acid tryptophan. Melatonin was first discovered and isolated in 1960[50], and later found to play roles in circadian rhythm and antixodiation[51,52]. Melatonin has multiple functions, acting both as an antioxidant and through receptor-mediated signaling.

Melatonin is primarily known for being synthesized in the pineal gland, which cyclically regulates serum melatonin levels according to a light/dark pattern [53]. However, intracellular levels of melatonin do not follow this same circadian pattern [54]. The highest concentration of extrapineal melatonin in the body is found in the brain, and within the cell it is highest in the mitochondria [54,55]. While the majority of melatonin is synthesized in the pineal gland, early research estimated about 20% of melatonin is synthesized extrapineally [56]. Recent work in the Friedlander laboratory found that this extrapineal melatonin is synthesized in the mitochondria [manuscript in preparation]. Cultured neural stem cells have melatonin present, and the amount of melatonin increases with differentiation, supporting the hypothesis that neuronal cells may produce melatonin [57].

In rats and humans, levels of melatonin decrease with age. In humans, the serum melatonin levels peak during childhood, and a 3.5 fold reduction was seen between age groups 35-50 years and 70-90 years of age [58]. Beyond this normal physiologic reduction with age, plasma melatonin levels in HD patients are sharply reduced and progressively decreases with disease [59,60]. This corresponds with sleep disturbances noted in 60-80% of HD patients [61].

In mouse models, melatonin production can vary. Pineal melatonin production follows circadian cycling in CBA and C3H mouse strains [62]. C57Bl/6 mice are enzymatically compromised in their ability to synthesize melatonin [63], but still express low levels of serum melatonin [62]. The mechanism for this contradiction remains unclear.

The HD model R6/2 mice, which are bred on the B6CBA background, an F1 hybrid of CBA and C57Bl/6, have progressive disturbed circadian patterns, sometimes showing complete sleep-wake cycle reversal at late stages of disease [64]. While plasma melatonin has been shown to be altered in these mouse strains, intraneuronal melatonin has never been measured.

1.2.2 MT1 receptors

Melatonin signaling in mammals is mediated by two melatonin receptors, MT1 (officially MTNR1A, previously Mel1A) and MT2 (officially MTNR1B, previously Mel1B). The melatonin receptors are their own subgroup of G-protein coupled receptors. They consist of 7 transmembrane alpha helical segments transversing the membrane. Both receptors are found on the cell membrane [65] , and recent work shows they are also localized to mitochondrial membrane[65]. The human *MT1* gene is mapped to 4q35.1 [66] and the mouse *Mt1* is located on chromosome 8 [67].

MT1 and MT2 are high affinity binding receptors, with MT1 showing a stronger affinity to 125I-melatonin than MT2[68]. Melatonin binds the receptor, resulting in a conformational change

in G-alpha-I. This change results in inhibition of cAMP formation and PKA activity, as well as a decrease in CREB phosphorylation[69-71]. Additionally, through coupling with other G proteins, activation of MT1 results in activations of many other G proteins.

G protein coupled receptors were previously believed to function as monomers, but more recent work shows not only do they form dimers, but this may be the primary or only form of functioning[72]. However, the biological significance of these dimers is unclear. MT1 and MT2 form both hetero- and homodimers. Significantly, GPR50 also interacts with these proteins. GPR50 is a G protein of unknown function which is considered a "melatonin-related receptor"[73]. *GPR50* is mapped to the X chromosome, and shares 45% amino acid homology with *MTNR1A* and *MTNR1B*[74,75]. Notably, GPR50 forms heterodimers with both MT1 and MT2. This dimerization significantly decreases the ability of MT1 to bind melatonin[76]. The exact mechanism of this inhibition is unknown, but may be due to GPR50 preventing critical interactions of MT1 and other proteins, including recruitment proteins.

Expression of pineal melatonin receptors reduces 1.75 fold with human aging; a fold change is seen in early stage Alzheimer's disease, with a progressive decrease through late stage of the disease[77]. Similarly, a 50% reduction in pineal melatonin receptors is seen in Parkinson's disease[78]. Extrapineal melatonin receptor expression also shows a decrease with aging in rats [79]. In R6/2 mice, expression of MT1 but not MT2 is reduced with disease progression; this same pattern is also seen in post-mortem human HD brains[65].

In C3H mice, *Mt1* mRNA and protein levels show daily fluctuations, high during the day and low at night, opposite the pattern of melatonin. *Mt1* mRNA in these mice is expressed in the brain in striatum, substantia nigra, and ventral tegmentum [80].

Therapeutic use of melatonin is considered very safe, including for long term use and during pregnancy [57]. Both melatonin and melatonin receptor agonists are available currently, with few known side effects. Agomelatine, a receptor agonist, is approved for treatment of depression in Europe; ramelteon, another receptor agonist, is approved in the U.S. for treatment of insomnia. Over-the-counter melatonin is available as a supplement in the U.S., and an extended release melatonin formulation, Circadin, is available in Europe [81]. Exogenously administered melatonin has a half-life of about 30 minutes [54].

1.2.3 Melatonin receptor mice

Mouse models are used extensively to study various roles of the melatonin receptors. MT1 knockout mice have decreased REM sleep time, with MT1 and MT2 playing complementary but distinct functions in sleep cycles[82]. Another knockout model showed a loss of blood glucose cycling rhythm, indicating the time dependent regulatory functions of MT1 extend beyond sleep[83]. Knockout of MT1 also results in an increase in intraocular pressure, death of retinal ganglion cells and increased reduction of photoreceptors with age[84,85]. Additionally, a knockout MT1 mouse model showed sensorimotor defects and an increase in depression, indicating MT1 plays a significant role in behavioral outcomes[86]. While studies with knockout MT1 mice have been performed in a variety of contexts, no previous studies have been done overexpressing the MT1 gene.

The role of MT1 in neuroprotection is subject to contradictory evidence. A study using MT1/MT2 double knockout mice treated with melatonin showed no protective effect on ischemic brain injury, suggesting the receptors were not the mechanism for melatonin's neuroprotection[87]. However, work in the Friedlander laboratory found melatonin's mechanism to specifically be

through MT1 *in vitro*[65]. To address these contradictions and clarify MT1's role in neuroprotection, more data is needed.

Therefore, in order to better understand the role of MT1 in neuroprotection, the Friedlander laboratory developed a transgenic mouse to overexpress MT1. The transgene consists of a human MT1 receptor gene, which is attached to a FLAG tag and EGFP sequence at the 3' end and controlled by a neuron-specific enolase promoter (NSE) (see figure 1). The NSE promoter expresses the transgene in primarily neurons; the promoter can also express sporadically in testes and kidneys[88,89].

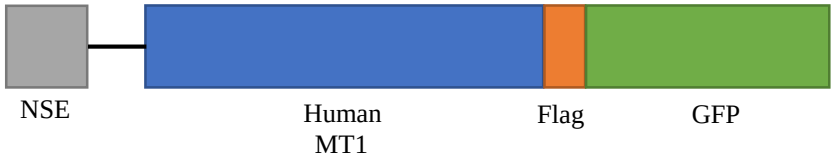

Figure 1. Transgenic NSE-MT1 mouse construct

Two founder mice were created for this transgenic model, labeled 2604 and 2606, on a B6CBA background. Both lines express the transgenic NSE-MT1 mRNA [personal communication, Yalikun Suofu, University of Pittsburgh]. My work uses the 2604 line, which is a more prolific breeder.

Characterization of the MT1 transgenic mouse is ongoing. Both body weight and brain weight are comparable to wild type B6CBA mice. Their brain volume is the same as that of wild type mice and they show no changes in number of cortical or motor neurons [personal communication, Amanda Mihalik, University of Pittsburgh]. NSE-MT1 mice kept for breeding had a lifespan of 18-24 months, similar to that expected of wild type B6CBA.

The NSE-MT1 mouse was developed on the B6CBA background specifically so that it could be crossed with the R6/2 mouse for HD research. The use of both the R6/2 and NSE-MT1 mouse models allows for an excellent method to study the role melatonin and melatonin receptors play in ameliorating the pathology of HD.

1.3 PREVIOUS STUDIES

Apoptosis and mitochondrial dysfunction are important components of neuronal death in neurodegenerative diseases. As a result, they provide a promising target for therapeutic drugs. In order to screen for possible therapeutic drugs, the Friedlander laboratory used the Neurodegeneration Drug Screening Consortium of 1040 compounds, a library of drugs put together by NINDS[90]. All of the compounds within the library are already FDA approved, making them ideal for therapeutic repurposing. These drugs were initially screened using isolated mitochondria, creating a cell-free method of screening. Follow-up screening was conducted in whole cell models.

The screened compounds were narrowed down by several criteria: ability to inhibit cytochrome c release, ability to inhibit cell death, and limited cell toxicity[90]. They were compared against the antibiotic minocycline. Minocycline inhibits the release of cytochrome c and has a neuroprotective effect in models of Huntington's, Parkinson's, ALS, and stroke[91]. From this screening, sixteen compounds, including melatonin, were selected for further testing[92].

Using primary cortical neurons (PCNs) exposed to oxygen glucose deprivation (OGD), which induces the release of cytochrome c and the activation of caspase-3, the Friedlander group quantified the protective effects of melatonin[92]. They then compared the protective effect against

H_2O_2 and NMDA induced cell death. In all of these cases, melatonin was effective in protecting against cell death[92]. It reduced overall death, as well as reduced apoptotic specific mechanisms of nuclear fragmentation and chromatin condensation.

To further understand the mechanisms of this protection, the researchers looked specifically at the release of cytochrome c and AIF from the mitochondria. Melatonin was able to block the release of these molecules, as well as inhibit the subsequent activation of caspase-3[92]. Melatonin was also able to inhibit the release and activation of caspase-1[92].

Additionally, melatonin was able to inhibit the loss of mitochondrial membrane potential resulting from OGD in PCNs[92]. This indicates that the mechanism involved is not just inhibiting the release of molecules from the mitochondria, but also functions by maintaining normal cellular function.

However, in studying mitochondrial permeability, it was found that melatonin does not reduce or inhibit this. It also did not have an effect on mitochondrial membrane potential in isolated mitochondria.

The Friedlander laboratory previously investigated the role melatonin receptors play in HD models of neurodegeneration. Cytochrome *c* colocalization was looked at in cell cultures of striatal cells ST14A with mutant *HTT*. When the cells were put into nonpermissive conditions (a shift from 33°C to 37°C), they released cytochrome *c* from the mitochondria. When these cells were treated with melatonin, the release of cytochrome *c* was inhibited. Melatonin also was able to partly inhibit the release of SMAC and AIF[65].

The Friedlander researchers further investigated the role melatonin receptors play in neuroapoptosis. Luzindole is a melatonin receptor antagonist and 2-iodomelatonin is an agonist. Using these molecules, they were able to determine that the neuroprotective effect of melatonin

requires binding to the melatonin receptor[65]. Additionally, knockdown of MT1 resulted in increased cell death; conversely, an increase in MT1 in cell models resulted in neuroprotection[65].

Progressing to in vivo models of HD, the research group found that administration of exogenous melatonin extended survival, delayed disease onset, and slowed disease progression within the R6/2 mouse model of HD. Additionally, it was found that the levels of MT1 are decreased in the brains of these mice, although this depletion can be ameliorated with the addition of exogenous melatonin[65].

1.4 CURRENT RESEARCH AND EXPERIMENTAL RATIONALE

Previous research shows a complex interplay between mitochondria, melatonin, and MT1 as they contribute to neuroprotection. My work aims to further understand the interactions of melatonin and MT1 and how this can be used to understand neuroprotection in HD.

Using the Friedlander generated transgenic MT1 overexpressing mouse and the well-known R6/2 mouse model, I determined whether increasing melatonin signaling via the MT1 receptor would further ameliorate symptoms and pathology of the HD model.

The R6/2 mouse's modeling of HD is characterized in several ways and my work utilizes each of these methods in order to determine the manner and extent of MT1 overexpression effects. To test the hypothesis, my work follows these aims:

1. <u>To determine whether melatonin is synthesized in neuronal mitochondria</u>

   Previous research shows MT1 to be localized to the mitochondria[65] and recent data demonstrate the neuronal mitochondria contain the terminal two enzymes of the melatonin synthesis pathway [manuscript in preparation]. While most plasma melatonin

is known to be synthesized in the pineal gland, the putative synthesis of neuronal melatonin in mitochondria has not been demonstrated.

2. To determine the effects of mutant huntingtin on endogenous and transgenic MT1

   MT1 is localized to the mitochondria and previous work by the Friedlander group shows that mutant *HTT* negatively impacts mitochondrial function[93]. Expression of MT1 is decreased in mutant-*HTT* cell models; treatment with melatonin significantly ameliorates the loss of MT1 protein[92]. Increased MT1 has a neuroprotective effect in cell models[65]. Adequate presence of MT1 is necessary for the protective effect of melatonin on the HD mouse[65]. Here I aim to test whether the HD phenotype specifically affects the expression of MT1, both endogenous and transgenic.

3. To determine the neuroprotective effects of melatonin via the MT1 receptor in an HD mouse model

   The previous studies done by the Friedlander group demonstrated a neuroprotective effect of melatonin both *in vitro* and *in vivo*. Further, they illustrated that this effect was mediated *in vitro* by MT1. This study aims to further understand how increasing melatonin signaling via MT1 may further ameliorate symptoms and pathology of the HD model.

1.5 PUBLIC HEALTH SIGNIFICANCE

Huntington's disease is a devastating and fatal neurodegenerative disease for which there are currently no available treatments. HD patients have a variety of options to treat individual symptoms, but no drug is currently available to treat the disease itself [94,95]. An estimated 30,000

individuals in the U.S. are living with HD[5]. Clinical genetic testing is available which allows an individual to know their genetic status, but only about 10-20% of those at risk get the testing done[96]. Those who test positive have no options for treating the disease they learn is inevitable for them.

While the cause of the disease, a CAG expansion in the *HTT* gene, has been known for over 20 years, many of the mechanisms of the disease itself remain unclear[3]. It is important to investigate both potential treatments for the disease as well as understand the ways in which these may modify the disease.

Melatonin has been shown to improve outcomes in neurodegenerative diseases such as stroke and ALS. In an HD mouse model, melatonin has been shown to delay disease onset, slow progression, and extend survival[65]. Melatonin as a therapeutic agent is safe for long term use and shows very few side effects [57,81]. Melatonin and melatonin agonists have been approved in both U.S. and European markets for treatment of sleep disorders and depression[81].

While the therapeutic use of melatonin has been studied across major neurodegenerative disorders, the mechanism of its neuroprotection remains unknown. Understanding mechanism of melatonin can help development of melatonergic drugs in treatment of Huntington's disease and other neurodegenerative diseases.

2.0 MELATONIN SYNTHESIS

2.1 INTRODUCTION

While most mitochondria is produced in the pineal gland, about 20% is produced extrapineally[56]. The synthesis and location of this extrapineal melatonin has not been extensively studied. However, recent research shows that in brain lysates, there is a particularly high concentration of melatonin compared to other tissues[65]. Additionally, at a cellular level, there is a high concentration of melatonin the mitochondria[97]. Melatonin has been shown to protect neurons from cell death *in vitro*; additionally, exogenous melatonin is neuroprotective in several *in vivo* models of neurodegeration including ischemic stroke, Parkinson's disease, ALS, and HD[90,92,98-100]. Neuronal death in HD is believed to be mitochondrially mediated[90]. Before further investigating the role of mitochondria in the neuroprotective effects of melatonin, it was therefore important to understand how melatonin and mitochondria relate.

Melatonin is synthesized from serotonin, via a multi-step enzymatic process. Serotonin is first converted to N-acetyl serotonin by the enzyme aralkylamine N-acetyltransferase (AANAT). Next, N-acetyl serotonin is synthesized into melatonin via the enzyme acetylserotonin O-methyltransferase (ASMT, also known as HIOMT, hydroxyindole O-methyltranferase)[101]. In rodents, non-human primates, and humans, these melatonin synthesis enzymes follow a cyclical pattern of expression in the pineal gland, with AANAT being the rate-limiting enzyme which controls this timed cycle[102]. Research in the Friedlander laboratory identified these two enzymes are localized to neuronal mitochondria [manuscript in preparation]. This mitochondrial

localization was seen across mouse, primate, and human brain samples. Notably, these enzymes do not appear to be light sensitive or cyclical in their expression.

With melatonin and its synthesizing enzymes localized to the mitochondria along with MT1, it was important to test whether these enzymes functionally produced melatonin within the mitochondria where it may directly bind the melatonin receptors. Melatonin was localized to the mitochondria via ELISA, but this method could not determine whether the melatonin was produced elsewhere and imported or synthesized in the mitochondria itself. To address this question, I collaborated with the University of Pittsburgh Small Molecule Biomarker Core to develop a novel method for measuring melatonin synthesis within the mitochondria. Here I use qualitative mass spectrometry to detect the synthesis of intermediate N-acetyl serotonin as well as the final melatonin product in isolated mouse brain mitochondria.

2.2 METHODS

For each repeat, 5 wild type mouse forebrains were pooled for mitochondrial isolation. The tissue was homogenized with a Teflon homogenizer and spun for 3 minutes at 1300g at 4°C. The pellet was resuspended and spun at the same conditions. The supernatant of each spin was combined and spun for 10 minutes at 21,000g at 4°C and used to isolate synaptosomal and non-synatosomal mitochondria according to published methods[93,103,104].

Mitochondria (synaptosomal and non-synaptosomal) (1μg/μL) were incubated in a respiration buffer (5 mM HEPES-Tris, 125 mM KCl, 2 mM Pi, 3 μM EDTA, 1 mM MgCl2, 1 mM ATP, 5 mM glutamate/malate, 5mM succinate, 10 μM deprenyl) with 50μm d4-serotonin for 30 minutes at room temperature, then quenched with sodium hydroxide. For the quantitative

experiments, 1ng/ml agomelatine (Sigma A1362), an MT1 agonist with a similar structure to melatonin, was added to act as an internal standard. The solution was extracted into chloroform at 5x reaction volume; samples were vortexed to maximize extraction efficiency, centrifuged (1000g for 5 minutes), and the resulting non-organic layer discarded.

Via a collaboration with the laboratory of Samuel Poloyac through the Small Molecule Biomarker Core, the samples were analyzed with ultra performance liquid chromatography mass spectrometry (Thermo Fisher TSQ Quantum Ultra) for the presence or absence of deuterated melatonin. Transitions used for analysis are 237 → 178 for d4 melatonin and 181 → 164 for d4 serotonin. Neat solutions (20 μM) of d4-serotonin, d4-melatonin (66521-38-8, AlsaChim), and n-acetylserotonin (1210-83-9, Sigma) were run to verify retention times and optimize detection. Mitochondrial samples were incubated with either respiration buffer alone or with deuterated serotonin. Respiration buffer with and without deuterated serotonin was also run to verify that deuterated serotonin did not spontaneously generate deuterated melatonin in the absence of cellular material.

The mass spectrometry output data were assessed for presence or absence of deuterated N-acetyl serotonin and deuterated melatonin.

2.3 RESULTS

To determine whether melatonin was synthesized in neuronal mitochondria, I incubated mitochondria (synaptosomal and non-synaptosomal) with deuterated serotonin. Because the deuterium labeled region of the serotonin molecule is not modified in the process of synthesizing melatonin from serotonin, any resulting deuterated melatonin indicated the ability of the isolated

mitochondria to perform this synthesis. Indeed, in wild type mouse mitochondrial samples, the d4-melatonin metabolite as well as the intermediate molecule, d4-n-acetly-serotonin were detected from samples incubated with d4-serotonin (figure 2). Demonstrating that this outcome is not the result of natural degradation of serotonin, samples with d4-serotonin alone, without the presence of mitochondria, showed no resulting d4-melatonin.

The results of the qualitative mass spectrometry provided a solid result indicating that melatonin is synthesized in mitochondria. The enzymes for this synthesis are localized to the mitochondria and are functional.

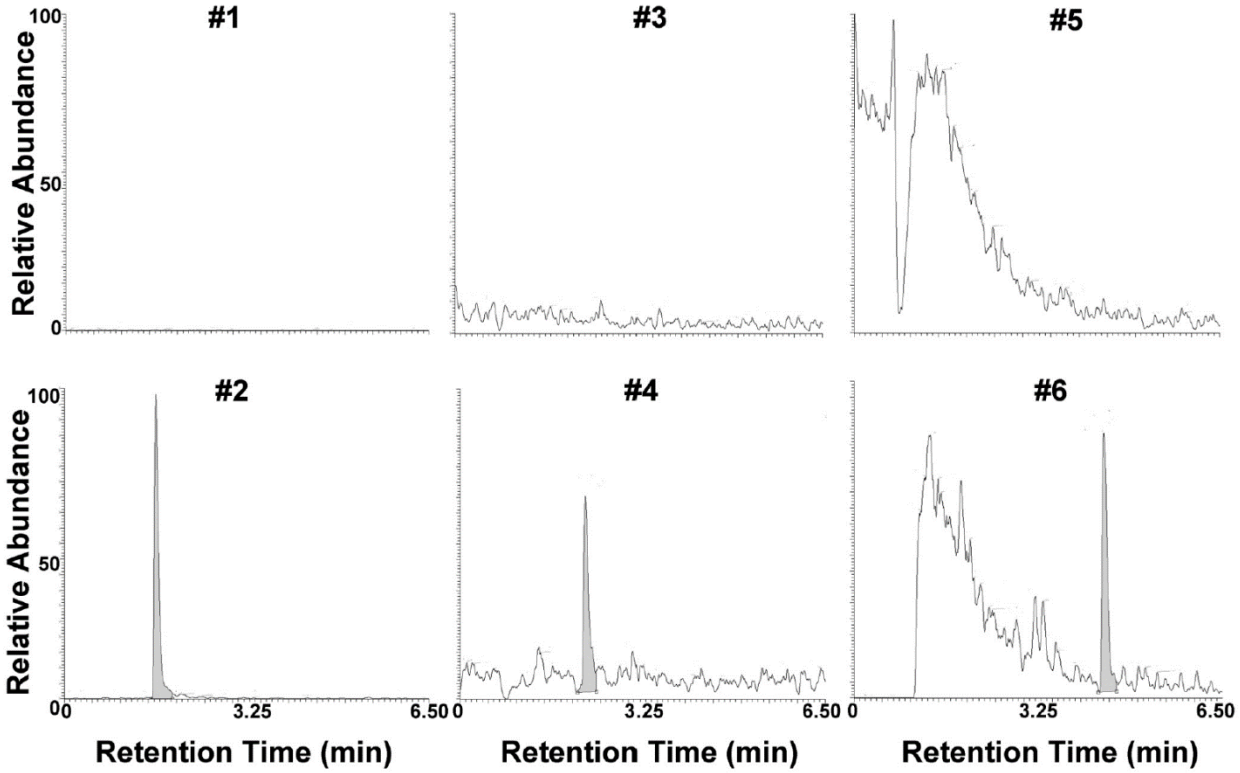

Figure 2. Melatonin synthesized from serotonin in neuronal mitochondria detected by mass spectrometry. Representative chromatograms showing metabolites extracted from mitochondria incubated in control buffer (#1,3,5) or with deuterated (d4) serotonin (#2,4,6). Samples incubated with buffer alone show no presence of d4-labeled metabolites (#1,3,5). Samples incubated with d4-serotonin show detected levels of unprocessed d4-serotonin (#2, retention time 1.65 minutes), d4-n-acetyl-serotonin (#4, retention time 2.28 minutes), and d4-melatonin (#6, retention time 4.45 minutes). This experiment was repeated three times and this figure is a representative result. For 1a-d, brain mitochondria were isolated from four mice per experiment, N=3.

2.4 DISCUSSION

Melatonin protects neurons from cell death in HD through a mitochondrially-mediated mechanism. Preliminary data from others in the Friedlander laboratory indicated the enzymes needed to synthesize melatonin from serotonin were present in the mitochondria. With this in mind, my work aimed to determine whether neuronal mitochondria synthesized melatonin, where it could directly modulate cell death pathways. Here I utilizes a novel method of detecting melatonin synthesis to demonstrate the AANAT and ASMT enzymes localized to the mitochondria are functional and melatonin can be fully synthesized from serotonin in mitochondria. The data establishes the first known autocrine signaling loop within the mitochondria. Neuronal melatonin is synthesized in the mitochondria, where it can immediately bind to its receptors without transport or release from the cell. These data also illustrate the central role of mitochondria in melatonin signaling, creating an important foundation for understanding melatonin receptor-mediated neuroprotection.

3.0 MT1 EXPRESSION

3.1 INTRODUCTION

Expression of human pineal melatonin receptors has been shown to decline with age, as well as in Parkinson's disease and Alzheimer's disease [77,78]. Extrapineal melatonin receptor expression has also been shown to decrease with aging in rats [79]. In the brains of R6/2 mice, MT1 is decreased as compared to wild type, although this depletion can be rescued by treatment with exogenous melatonin[65]. This rescue capability suggests the presence of a positive feedback mechanism with melatonin levels having a positive correlation with MT1 levels. It is also clear that the HD disease phenotype critically interacts with MT1 expression. My aim here is to characterize how the phenotype of standard R6/2 mice affects the expression of both endogenous and transgenic MT1. Previous research on MT1 expression in R6/2 mice was done on a unique version of the model with a longer CAG repeat length, which lead to atypical disease progression; I sought to replicate the reduction of MT1 in the traditional Jax strain of R6/2, with CAG length consistent with the original publication of the strain. Furthermore, I sought to determine whether any reduction in endogenous MT1 expression would be mirrored in expression of the transgenic MT1. To this end, I examined the expression of endogenous and transgenic MT1 in wild type, R6/2, and NSE-MT1-R6/2 mice in late stage disease to determine effects of the R6/2 phenotype on both types of MT1 expression.

3.2 METHODS

The University of Pittsburgh Institutional Animal Care and Use Committee approved all experimental procedures, protocol #15015004. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For both mRNA and protein studies of endogenous MT1 expression, wild type and R6/2 mice were used. Each sample consists of 5 mice brains pooled. The resulting material was then divided between use for RNA isolation, whole brain protein collection, and isolation of and protein collection from synatosomal and non-synaptosomal mitochondria. Mice were studied at 9 and 12 weeks of age (mid and late state disease) with an n=3 for each time point (a total of 15 mice per age per genotype).

For study of transgenic MT1 expression, NSE-MT1 and NSE-MT1-R6/2 mice were used. Experimental mice where offspring from a cross of female NSE-MT1 transgenic mice [B6CBA-Tg(NSE-MTNR1A)/Rmf(Pitt)] and R6/2 males [B6CBA-Tg(HDexon1)62Gpb/3J (Jackson Laboratory)]. All mice were bred in triads, which increased rates of pregnancy resulting from each R6/2 breeder. CAG length of R6/2 mice was verified for each breeder and animals outside of the accepted 150-160 repeat range were excluded from breeding and experiments. Wild type littermates were used during optimization and verification. Individual mice were used for n=3 per genotype, with brains collected at 12 weeks of age.

3.2.1 Expression at mRNA level

Whole brain was homogenized in mitochondrial isolation buffer at 4°C. Total RNA was isolated by taking 25µL of this whole brain homogenate for lysis in 500µL TRIzol reagent [Life

Technologies, 15596-026] and incubated 5 minutes at room temperature. 100μL of chloroform was added, tubes shaken and incubated at room temperature for 3 minutes. Samples were at 12,000g for 15 minutes at 4°C and aqueous phase containing RNA transferred. RNA was precipitated with 500μL isopropanol per sample and incubated at room temperature for 10 minutes, then centrifuged at 12,000g for 10 minutes at 4°C. Supernatant was removed and RNA washed in 75% ethanol before being resuspended in nuclease free water. RNA was treated with a DNase kit [Invitrogen, 18068-015]. After DNase treatment, RNA was purified by incubation with 0.1 volumes 3M sodium acetate and 3 volumes cold 100% ethanol and incubated overnight at -20°C, followed by 30 minute centrifugation at 4°C at 13,000 rpm and two washes with 75% ethanol, and finally resuspended in nuclease free water. RNA quality and quantity were determined using the Take3 Micro-volume plate system on Biotek Synergy plate reader. All isolated mRNA had a 260/280 ratio of at least 1.8. Generation of cDNA was using High-Capacity cDNA Reverse Transcription Kit [Applied Biosystems, 4368814] following manufacturer instructions.

QPCR for wild type and R6/2 samples was run using primers for endogenous MT1 mRNA [Qiagen PPH02532A-200] using Power SYBR Green PCR Master Mix (Applied Biosystems). Reaction volume totaled 15ul and all reactions were run in triplicate. Reaction mix and cycle conditions followed manufacturer protocol.

QPCR to compare MT1 and MT1-R6/2 samples was run using primers designed to specifically detect the transgene. The forward primer site is within the MT1 portion of the gene while the reverse primer site is within the GFP sequence [Forward: TGGCCGATAGGGTTAAATGGA, reverse: ACCTTGTCGTCATCGTCTTTG]. These samples were run with conditions: 95°C for 5 minutes, followed by 40 cycles of 30 seconds at 95°C and 30 seconds at 62°C.

For all sets, RN18S (housekeeping) was used for normalizing results [forward: CGTAGTTCCGACCATAAACGATG, reverse: GCTATCAATCTGTCAATCCTGTCC].

Results were read using BioRad CFX Manager software and analyzed by computing relating MT1 expression normalized to RN18S according to protocol from Livak and Schmittgen[105]. NSE-MT1 ΔΔct values were divided by WT to determine fold change; fold changes of each sample set were averaged and transformed by normalizing WT to 1. The same process was applied to determine normalized fold changes for NSE-MT1-R6/2 compared to NSE-MT1.

3.2.2 Expression at protein level

The protein levels of endogenous MT1 were assessed in WT vs R6/2 mouse brains. Although the cause is unclear, the transgenic MT1 protein in the 2604 MT1 mouse line cannot be detected; for the purposes of this dissertation only endogenous MT1 was quantified at the protein level.

Protein levels of MT1 were assessed in whole brain homogenate and mitochondria (nonsynaptsomal and synaptosomal). Mitochondria were isolated as described in section 2 of this dissertation. Whole brain homogenate and isolated mitochondria were incubated with RIPA buffer at 5x volume on ice for 30 minutes to lyse tissue. Samples were mixed with 4X SDS sample buffer [Boston Bioproducts, BP-110NR] and denatured at 55°C for 15 minutes. All samples were run on SDS-PAGE, using a 10% acrylamide gel, then transferred to PVDF membrane [Sigma, Immobilon-FL, 05317]. Membrane was blocked using Odyssey blocking buffer [Li-Cor, P/N 927], incubated with MT1 antibody [Thermo Scientific, PA519109, 1:1,000] and for whole brain samples, β-actin antibody [Sigma, A5441, 1:10,000] or for mitochondrial samples, Cox I antibody [Santa Cruz, sc-19998, 1:1,000] at 4°C overnight, washed with PBST, and incubated with

appropriate secondary antibodies [Li-Cor IRDye, 1:30,000]. Blots were scanned on Odyssey CLx [Li-Cor] and band intensity quantified using Image Studio software [Li-Cor]. Intensity of each MT1 band was normalized by the intensity of respective loading control bands (β-actin for whole brain samples, Cox I for mitochondrial samples). Each R6/2 sample was compared against its respective WT sample from the same isolation and preparation to determine fold change. Fold changes for n=3 were averaged to determine final compared differences.

3.2.3 Statistical analysis

Analysis of each protein and mRNA pair of values was done using paired t-tests.

3.3 RESULTS

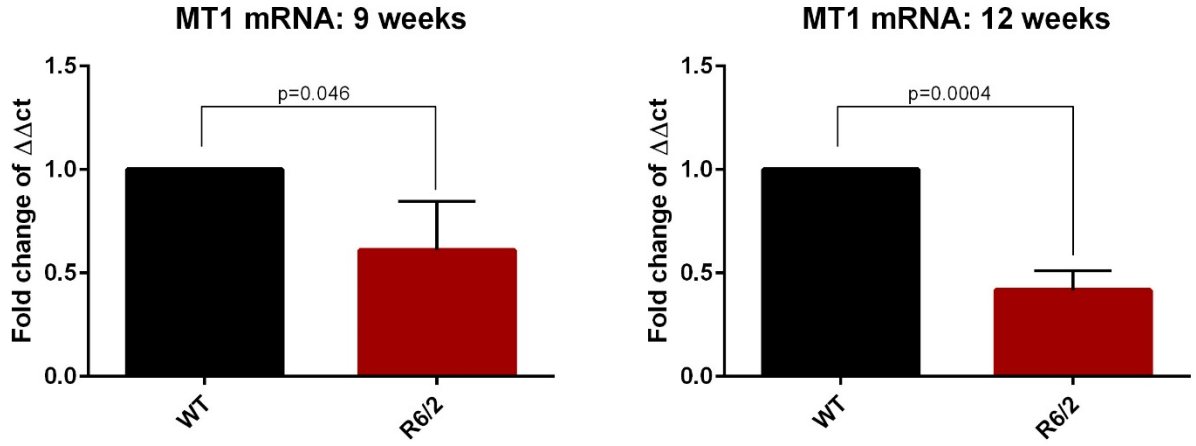

Figure 3. Endogenous MT1 mRNA expression significantly reduced in R6/2 mice at mid and late stage disease.
Changes in relative expression of endogenous MT1 mRNA shown as the fold change of ΔΔct for WT and R6/2 mice at both 9 weeks (left) and 12 weeks (right) of age. Three biological repeats were used consisting of 5 pooled brains each, with each of the three samples run in triplicate. Expression was normalized by housekeeping gene RN18S, then fold differences calculated. Significant differences at both timepoints determined by paired t-test.

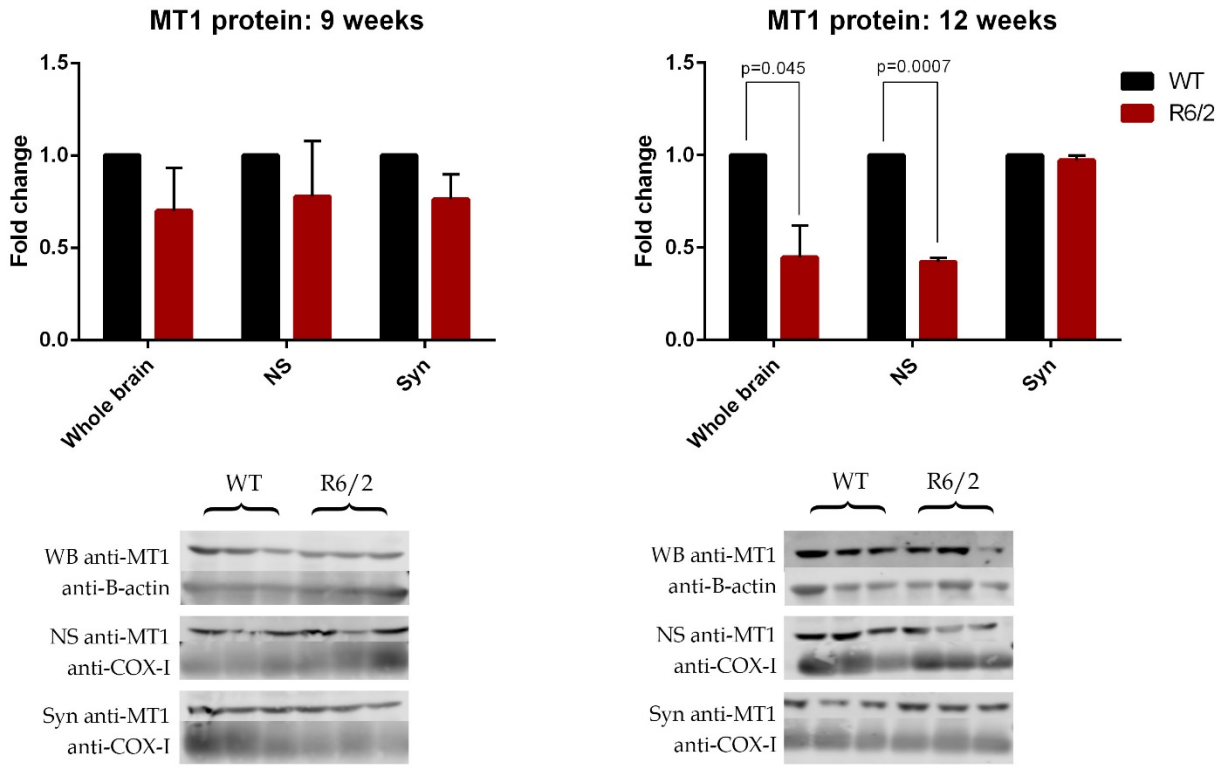

Figure 4. Changes in endogenous MT1 protein expression seen in whole brain and mitochondria in R6/2 at mid and late stage disease.
Quantified changes in MT1 protein expression in whole brain and non-synaptosomal (NS) and synaptosonal (Syn) mitochondria from WT and R6/2 mice at 9 week (left) and 12 week (right) timepoints. Three repeats of each sample were used, with each sample consisting of 5 pooled brains. Blots were probed with anti-MT1 antibody and appropriate loading control (B-actin for whole brain samples, Cox I for mitochondrial samples), with blot examples shown. No significant differences were detected at 9 weeks, but whole brain and non-synaptosomal mitochondria expression were significantly reduced at 12 weeks of age.

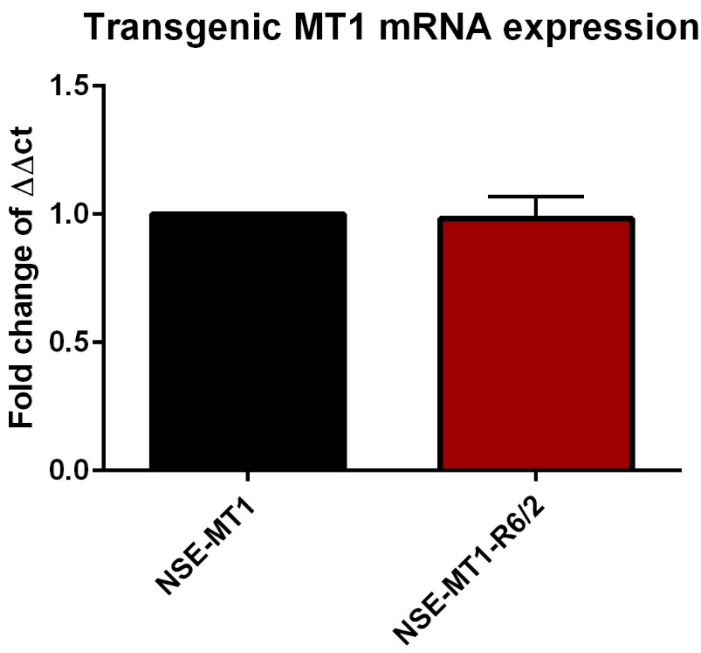

Figure 5. Expression of transgenic NSE-MT1 mRNA in NSE-MT1 and NSE-MT1-R6/2 mice at 12 weeks of age.
Three biological repeats were used with each sample run in triplicate. Expression was normalized by housekeeping gene RN18S, then fold differences calculated. Unpaired t-test showed no significant difference between the two (p=0.7564).

At 9 weeks of age, a reduction in MT1 expression can be seen at the mRNA level, with approximately a 40 fold decrease (p=0.046). At 9 weeks this change in the raw data is not significant at the protein level; additionally there are no significant changes in the mitochondrial protein level (figure 4). At 12 weeks of age, mRNA is significantly reduced even further (60% decrease, p=0.0004) (figure 3). Protein is also reduced in 12 week whole brain and non-synaptosomal mitochondria. This reduction is not seen in synaptosomal mitochondria (figure 4).

In mice with transgenic MT1, there is no significant difference in the NSE-MT1 mRNA expression with the addition of the R6/2 genotype (p=0.7564) (figure 5).

3.4 DISCUSSION

Previous research within the Friedlander group showed a decrease in MT1 protein expression in both whole brain and mitochondria in the extended CAG repeat R6/2 model. Here, I demonstrated that a similar reduction in standard Jax R6/2 mice. Additionally, I characterized the expression at an mRNA level. The mRNA is decreased at both 9 weeks (mid-stage disease) and 12 weeks (late-stage disease). While the protein in mitochondria appears slightly decreased in R6/2 across whole brain and mitochondria at 9 weeks of age, this change is not significant until 12 weeks of age, where there is a significant decrease in both whole brain and non-synaptosomal mitochondrial protein. Interestingly, I detected no reduction of MT1 in synaptosomal mitochondria. Synaptosomal mitochondria are isolated from the active synaptic terminal of neurons. These data may suggest the reduction in MT1 does not severely impact the active functional aspect of neurons; however, neuronal death can still be impacted by MT1 reduction elsewhere and receptor-mediated melatonin neuroprotection may function there. Alternatively, reduction of MT1 may increase synaptic neuron death or result in die-back of processes reducing the number of synapses overall even in the presence of surviving neuron cell bodies, resulting in elimination of synaptosomal mitochondria from the sample collected. Another possible explanation for the unchanged MT1 expression in synaptosomal mitochondria is the process of isolating these mitochondria may enrich healthy mitochondria, resulting in higher detection of MT1.

These data both verify the effects of the HD phenoytype on MT1 expression and, with the addition of the mRNA expression, provide insight that the effect takes place at or before the transcriptional level. No microRNAs known to interact with MT1 are upregulated in HD models, but there are many possible other mechanisms which may influence mRNA expression including promoter changes or transcriptional dysregulation.

The intention of the transgenic MT1 model for this project is to determine if MT1 reduction can rescue the R6/2 phenotype. Therefore it was critical to determine whether the R6/2 phenotype affected the expression of transgenic MT1 as well. With this data, I demonstrate that the R6/2 phenotype does not appear to affect transgenic MT1 mRNA expressions because the expression in the NSE-MT1 mouse alone equals the expression in the NSE-MT1-R6/2 mouse.

This provides a solid basis for using the NSE-MT1 mouse model crossed with the R6/2 mouse to determine whether overexpression of MT1 can improve the neuroprotective effects of melatonin in that model.

4.0 BEHAVIOR AND SURVIVAL

4.1 INTRODUCTION

An advantage of the R6/2 model of Huntington's disease is that it shows progressive motor defects that can be characterized by an array of standard mouse behavioral tests[106]. While the characterization of disease symptoms in mouse models can sometimes be difficult due to the way that motor, cognitive, and psychiatric symptoms can interact and overlap, there is a distinct motor deficit progression that can be measured in the R6/2 mice[44,106]. Various tests are available, but one consistently used for R6/2 mice is rotarod. Critically, fixed speed rotarod was used for the initial work showing melatonin's neuroprotective effects in the R6/2 mouse, and this set of experiments aims to repeat that methodology for consistency in both generating and understanding outcomes[65].

Because the MT1 transgenic model is newly developed, it was characterized with rotarod testing before being used for these experiments, to rule out any effects of the transgene on behavioral performance. The MT1 transgenic performed equally well as wild type littermates [personal communication, Amanda Mihalik, University of Pittsburgh]. Additionally, lifespan of MT1 transgenics appears to be normal. Survival studies have not been specifically performed, but through colony maintenance within the Friedlander laboratory breeders had a natural survival of 1-2 years, the typical lifespan for wild type mice of this background.

For this study, mice were tested on fixed speed rotarod at two speeds. The higher speed, 15rpm, is used as an indicator of disease onset, with mice beginning to show decreased latency to

fall as motor symptoms first appear. The lower speed, 5rpm, is used as an indicator of disease progression, showing the ongoing progressive decline in motor ability.

Additionally, this study looks at changes in mouse survival. Survival time of the R6/2 mice has been found to be a good indicator of neuroprotection[45].

The study uses F1 offspring from crosses of R6/2 and MT1 mice. This cross results in 4 potential genotypes: NSE-MT1-R6/2 (R6/2+, MT1+), R6/2 (R6/2+, MT1-), MT1 (R6/2-, MT1+), and wild type (R6/2-, MT1-). Mice without R6/2 genotype (including both wild type and MT1 transgenic) were used as controls for normal performance, but not used for analysis. The aim of this set of experiments was to determine whether an increase in melatonin signaling via the MT1 receptor will further ameliorate the disease phenotype in R6/2 mice with respect to clinical measures.

4.2 METHODS

The mice used for this set of experiments were the offspring of NSE-MT1 transgenic [strain B6CBA-Tg(NSE-MTNR1A)/Rmf(Pitt)] females, supplied from a colony I bred and maintained, and R6/2 males, supplied from our laboratory's continuously maintained colony. Due to the disease phenotype, female R6/2 mice have very low fertility and we observe when litters are born, pups are small, and the females do not care for the pups. While our R6/2 males also show lower rates of fertility compared to WT males, they are still able to generate offspring [personal communication, Lisa Ferrando, University of Pittsburgh]. The use of NSE-MT1 females for breeding ensured higher pregnancy rates, better litter sizes, and proper care of offspring. All mice were bred in triads, to increase chances of fertilization from each R6/2 male breeder.

The R6/2 strain [B6CBA-Tg(HDexon1)62Gpb/3J (Jackson Laboratory)] is a fragment model using human exon 1 containing pathogenic CAG repeat length. In the Friedlander colony, this polyglutamine repeat consists of 150-160 tandem CAG repeats. Repeat length is verified with site specific primers (see below) with PCR product run on 1% agarose gel and approximate base pair size measured using Invitrogen 50kb ladder [personal communication, Lisa Ferrando, University of Pittsburgh]. Mice outside of this range are excluded from breeding and experiments.

All of the F1 offspring of NSE-MT1 and R6/2 crosses were genotyped at age 15-25 days using two sets of primers. The first was with commonly used R6/2 primers CAG1 and Hu3, also used for R6/2 colony maintenance and repeat length measurements.

CAG1: 5'-ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC-3'

Hu3: 5'-GGCGGCTGAGGAAGCTGAGGA-3'

Second, the mice were genotyped with a set of transgenic MT1 primers which I designed. These primers amplify a section of the transgene which spans from the MT1 to GFP portions, eliminating the chance that the primers would pick up endogenous MT1 as well.

Forward: 5'-GTACGACAAACTGTACAGCAG-3'

Reverse: 5'-GAACTTCAGGGTCAGCTTGC-3'

Previous work has shown no difference in behavioral measures between male and female R6/2 mice[45]. For this experiment, I used all female mice because males were used for mouse colony maintenance. After genotyping, I weaned mice directly into their experimental cohorts with 4-5 mice per cage. A significant enrichment effect has been seen in the R6/2 mice when mutants are housed with wild type[107]. In order to avoid confounding due to this effect, all mice are housed only with other mice of the same genotype.

4.2.1 Treatment

I began melatonin treatment of 30mg/kg at 30 days of age and continued until death. The dose was chosen after preliminary experiments using 30, 50, and 100mg/kg doses demonstrated 30mg/kg to be a saturating concentration in R6/2 mice. Mice received intraperitoneal injections daily with a total injection volume of 100µl. Injection sites alternated between left and right sides of body each day to decrease irritation. Melatonin (Sigma, M5250) is not soluble in water/saline. The melatonin was first dissolved in DMSO and vortexed briefly; DMSO volume was calculated to 3% of total solution volume. Once melatonin was fully dissolved, PBS was added to bring the solution to its final volume.

Because daily handling and injection can cause stress in these animals and alter disease phenotype, control groups were also injected daily with 100µl vehicle.

Eight cohorts consisted of the following genotypes and treatments:

R6/2 mutant + MT1 mutant (melatonin; vehicle)
R6/2 mutant only (melatonin; vehicle)
MT1 mutant only (melatonin; vehicle)
Wild type (melatonin; vehicle)

4.2.2 Behavior analysis

Previous studies by the Friedlander group on R6/2 mice have used fixed speed rotarod [65]. The behavior measures for this aim were consistent with this in order to generate comparable data and allow for analyzing similarities or differences in trends with earlier characterizations of these mice.

The rotarod system (Columbus Instruments) measures active behavior, yielding results that indicate coordination, strength, and endurance. Each mouse was trained at 5 weeks of age on the fixed speed rod at 15rpm until able to complete a 7 minute trial without falling. Any mice unable to adequately perform during training were removed from test groups. Trial testing consisted of placing a mouse on the constant speed rotarod at 15rpm and 5rpm for 7 minutes each; mice are given three attempts to complete the trial without falling, with a minimum 10 minute rest between attempts. The best time from these three attempts was used for analysis. Mice who died before the end of rotarod trials were recorded at 0 minutes; these mice were not censored from the experiment to avoid weighting the average time towards longer living mice.

4.2.3 Survival

The same mice used for behavioral testing were also used for survival analysis. I monitored the mice daily and counted end of survival as either death or a mouse's inability to right itself within 30 seconds of being placed on its back, consistent with previous R6/2 work[45].

4.2.4 Statistical analysis

Rotarod times were compared week by week using Friedman's 2-way non-parametric ANOVA to compare timepoints between grouped cohorts (saline treated R6/2 and NSE-MT1-R6/2 compared to melatonin treated R6/2 and NSE-MT1-R6/2), which indicated significant differences between melatonin and saline treatments, starting at 10 weeks of age for 15rpm and 11 weeks of age for 5rpm. Kruskal-Wallis tests were used to compare timepoints between individual cohorts (separate genotypes and treatments): melatonin treated R6/2 with melatonin treated NSE-MT1-R6/2. A GEE

repeated measure analysis was used to analyze rotarod times as a whole for overall trends. Assistance in choice and performance of these statistical tests was provided by Yuefang Chang, PhD of the Department of Neurological Surgery.

The survival for R6/2 positive groups was compared using a log-rank test. Follow-up log-rank tests were used to evaluate the differences between melatonin and saline treatments for each genotype, using a Bonferroni-corrected threshold for determining p value significance (threshold=0.0083).

4.3 RESULTS

In order to establish the melatonin dose to be used for this set of experiments, I first tested 30, 50, and 100 mg/kg doses by injection. I found that in open field measurements of R6/2 performance, increased dose provided no improved protection, indicating 30mg/kg to be a sufficient saturating dosage. In addition, I found that while open field behavioral testing provided sufficient data to assess dosage, its outcomes were not comparable to the previously established rotarod protocol in the Friedlander laboratory; thus rotarod was used for subsequent studies.

Given three trials at each speed, the best time for each mouse was used for analysis. Times for each cohort were averaged. At 15rpm (figure 6), the speed indicating disease onset, the groups receiving saline treatment performed poorer than those receiving melatonin treatment ($p<0.0001$). Of the groups receiving melatonin, the raw data suggests that the NSE-MT1 transgenic performed better starting at 10 weeks; however the difference between the R6/2 melatonin treated and the NSE-MT1-R6/2 melatonin treated is only significant at 13 weeks ($p=0.004$). Both individual timepoints and overall trend were compared. While melatonin treated NSE-MT1-R6/2 was

significantly improved at the 13 week timepoint compared with melatonin treated R6/2, when the cohorts are analyzed as a whole, they are not significantly different (p= 0.180).

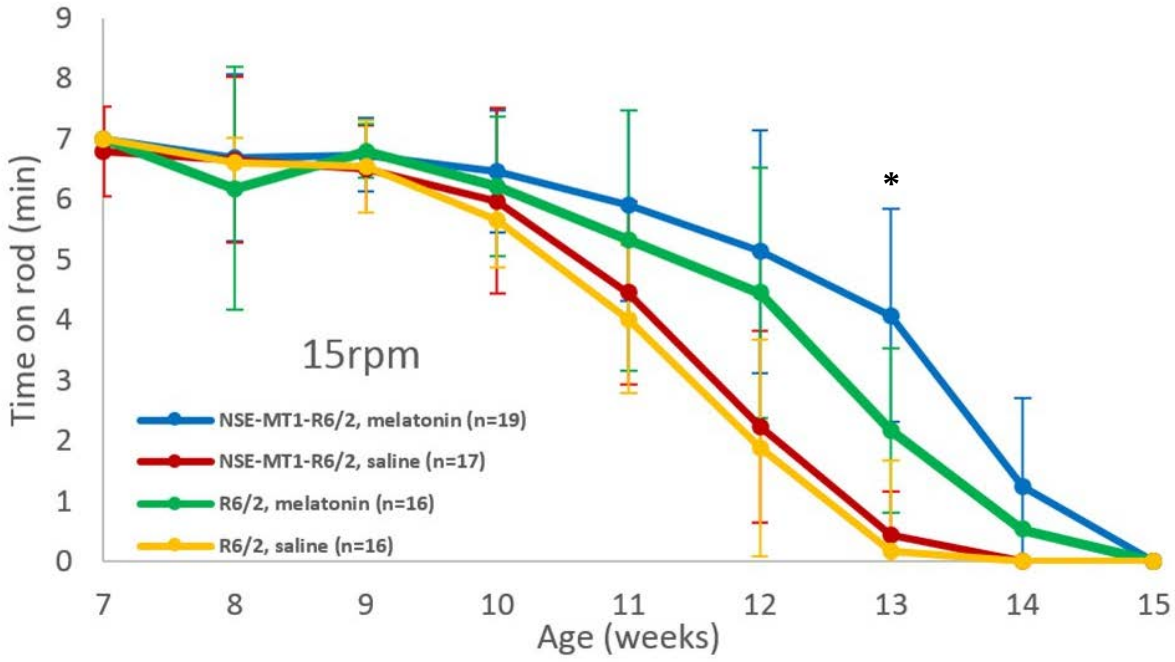

Figure 6. Time on rotarod at 15rpm for R6/2 cohorts, +/- MT1 transgene, +/- melatonin injection.
Mice were placed on the rod for trials of 7 minutes total with 3 attempts to pass. The maximum time each mouse stayed on the rod during the three trials was recorded. Performance at 15rpm is indicative of age of disease onset.

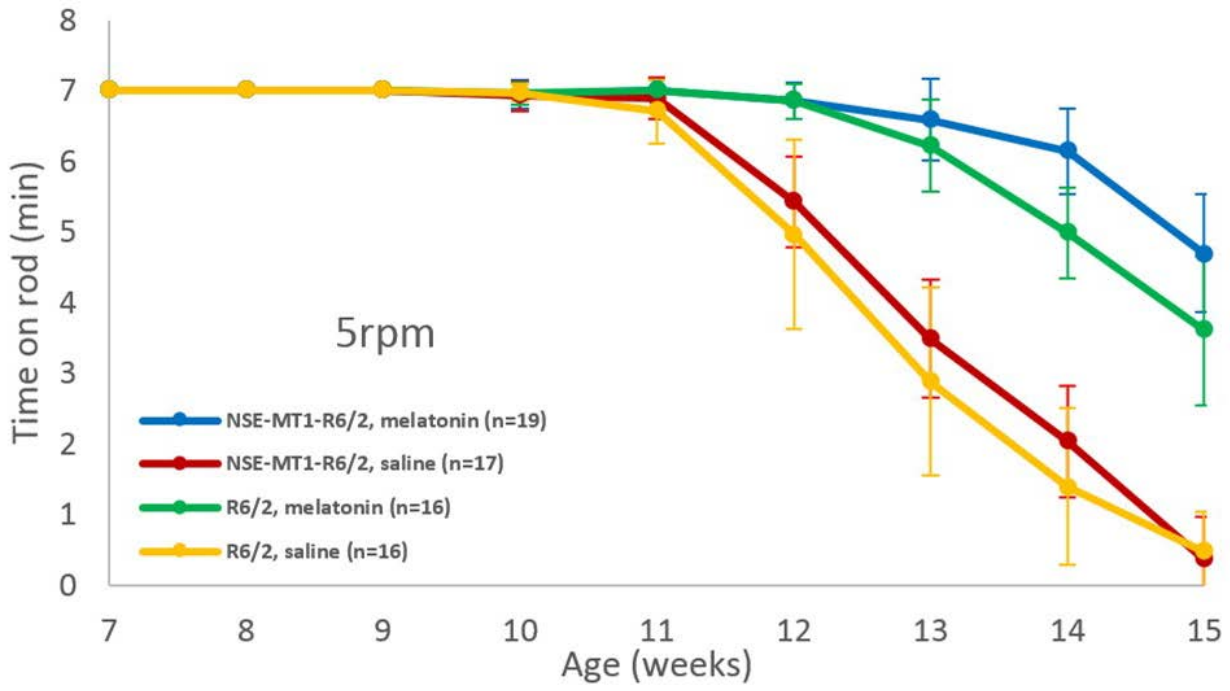

Figure 7. Time on rotarod at 5rpm for R6/2 cohorts, +/- MT1 transgene, +/- melatonin injection.
Once cohorts failed the full 7 minutes for 15rpm, they were additionally tested at 5rpm. Mice were placed on the rod for trials of 7 minutes total with 3 attempts to pass. The maximum time each mouse stayed on the rod during the three trials was recorded. Performance at 5rpm is indicative of disease progression.

At 5rpm (figure 7), the speed indicative of disease progression, a similar trend is seen as at the higher speed. The mice receiving melatonin treatment performed much better than those receiving only vehicle ($p<0.0001$). However, the difference between R6/2 with melatonin treatment and NSE-MT1-R6/2 with melatonin treatment is small. This difference is significant at 14 weeks ($p=0.0003$), but no other timepoints. The overall trend for these groups shows no significant difference ($p=0.150$).

Table 1. Summary table of average survival (days) for each cohort ± standard deviation.

	Saline	**Melatonin**
R6/2	97.0 ± 8.9	108.5 ± 5.8
NSE-MT1-R6/2	99.0 ± 9.0	112.0 ± 9.2

These mice were also assessed for survival (figure 8). Survival analysis showed a significant difference in median age of death (summarized in table 1) between saline vs melatonin treatment ($p<0.0001$ for NSE-MT1-R6/2 genotype, $p=0.0028$ for R6/2 genotype) but there is no significant difference between melatonin treated R6/2 vs NSE-MT1-R6/2 ($p=0.1053$).

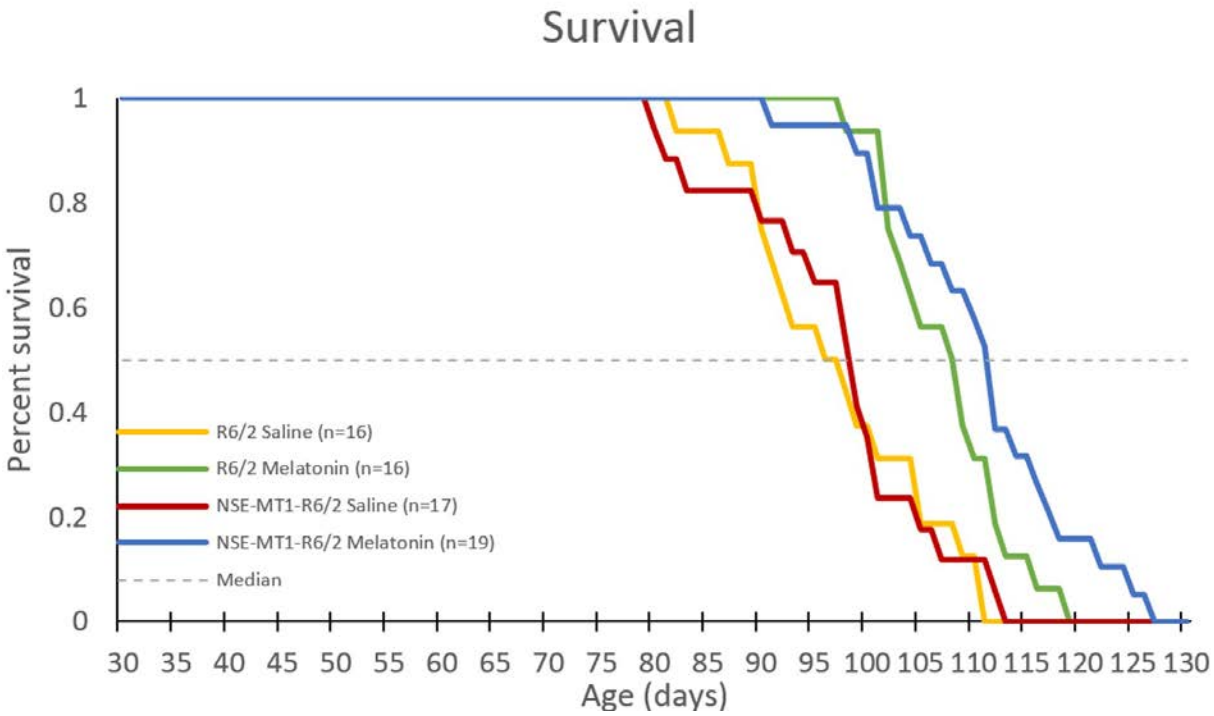

Figure 8: Survival of R6/2 mice +/- NSE-MT1 genotype, +/- melatonin treatment.
Survival was observed for the same cohorts of mice used in rotarod testing. Survival was defined as death or mouse's inability to right itself within 30 seconds when placed on its back.

4.4 DISCUSSION

As seen in previous work within the Friedlander laboratory, melatonin was beneficial in both behavioral measures and survival in the R6/2 mice[65]. However, contrary to the initial hypothesis, the behavior and survival in these melatonin treated R6/2 mice was not further extended with the

addition of MT1 overexpression. This result lends itself to several possible explanations which warrant potential further investigation.

4.4.1 Molecular influences

While cellular models indicated that melatonin neuroprotection was mediated through MT1, it is possible that in an *in vivo* chronic model of Huntington's, this protection may be affected by the chronic nature of the *in vivo* model, supplemented by an alternate mechanism, or modified through a regulatory mechanism.

Cellular models testing the mechanism of melatonin's neuroprotection studied an acute stress. Additionally, this MT1 overexpression transgenic has shown to additively protect against neuronal death in models of stroke [manuscript in preparation]. The stroke model represents another acute injury. Melatonin's neuroprotection appears to be primarily mediated by MT1 in such acute models; however, the MT1 mediated protection may not be the primary mechanism in a chronic model.

In this chronic model, the receptor-mediated mechanism of melatonin may be supplemented by melatonin's role as an antioxidant. In post-mortem human HD brains, several markers of oxidative stress are elevated[108]; a similar pattern is seen in the R6/2 model mouse as well[47]. The cellular damage caused by increased free radicals and oxidative stress may be a contributing factor to the HD phenotype. Therefore, melatonin's additional role as an antioxidant may confound these results investigating receptor-mediated protection. Melatonin may function as a neuroprotectant through both antioxidant and receptor signaling pathways, rather than MT1 alone.

Melatonin dosing may be another factor for understanding the experimental outcomes seen here. The melatonin dosage for these experiments was determined using R6/2 mice. This established 30mg/kg as a saturating dose for the R6/2 mouse model. However, because the dosage was not determined in the NSE-MT1-R6/2 mouse, we cannot be sure the 30mg/kg dose is saturating with the additional MT1 expression. If the melatonin was not saturating in these mice, we would not be able to see the full protective effect of melatonin with overexpressed MT1.

Finally, in discussing the biological function of MT1, it is vital to address the role of GPR50. GPR50, as a melatonin receptor-like G protein, is able to dimerize with MT1. This dimerization results in a significant reduction in MT1 signaling. While the function of GPR50 is yet undiscovered, it may play a role in regulating melatonin signaling. In the context of this work, it may affect the functionality of the transgenic MT1, reducing the overall impact of the overexpression.

4.4.2 Mouse model influences

While these results may indicate a number of possible outcomes regarding melatonin's mechanism of action and the role MT1 plays in neuroprotection, I must also consider that the results may be due to the model itself. While the transgenic MT1 has been detected to be overexpressed at the mRNA level [personal communication, Amanda Mihalik, University of Pittsburgh], the transgenic protein itself has been particularly difficult to detect and characterize. Ongoing work indicates that while it is overexpressed, the fold increase may be lower in the 2604 line used for these experiments compared to the second founder line, 2606.

As a result, this particular line of transgenic MT1 mice may not overexpress the receptor at a high enough level to effect significant change at a behavior or survival level. The ideal solution

to this potential flaw in the MT1 mouse model is to use a line with higher transgenic expression. The Friedlander laboratory maintains a second MT1 line from a second founder, which has shown higher mRNA expression as well appearing to have increased protein expression. However, consistent with studies which have shown MT1's impact on reproductive cycles and hormones[68,73], these mice breed very poorly, taking an extended period of time to generate pregnancies, with resulting litter sizes of only two to three pups. Pairing this very poor breeding line with the poor breeding R6/2 line was not considered feasible to generate significantly sized cohorts for such a study as this.

Further studies may benefit from additionally looking at the effects of MT1 knock-down/out. Several MT1 knockout (KO) lines have been developed, primarily for use in studying circadian rhythm. If the proposed mechanism is correct and the inability to detect significant behavioral changes is indeed due to the NSE-MT1 model itself, I would expect that R6/2 positive, MT1 KO crossed mice would produce a dramatically more severe phenotype, unable to be rescued by treatment with melatonin.

While this work is not feasible within the scope of this dissertation project, it represents an important follow-up experimental design when it comes to understanding molecular mechanisms when using genetic mouse models. MT1 KO lines are currently available commercially.

A second potential limitation of these mouse models is the rapid disease progression and short lifespan of the R6/2 mouse. R6/2 was the first HD mouse model developed[47]; however, it has its limitations as a model, most notably being a fragment and not full length model and being a very rapid and severe model of disease onset and progression, which led to the development of a plethora of other HD model mice[109]. R6/2 continues to be a widely used model, with over 500 publications to date using it, and it is a valuable tool for understanding the pathology of HD.

However, the behavior and survival data shown here indicate that with MT1 overexpression effect being seen only at very late timepoints (13-14 weeks), the mice may die before a significant effect can be observed.

The highest CAG repeat lengths in human HD are seen in juvenile HD, which typically has 60+ repeats[110]. With 150-160 repeats, the R6/2 model is markedly more severe. The R6/2 model in fact has the longest CAG repeat length of any HD mouse model and by far the shortest lifespan[109]. This short and severe phenotype is beneficial for extensive studies with a rapid turnover of animals with relatively little variability in survival. However, it may best be supplemented by a less severe model in order to fully understand the mechanisms of MT1-receptor mediated neuroprotection.

5.0 CASPASES AND HISTOLOGY

5.1 INTRODUCTION

The R6/2 mouse is noted for having progressive behavioral and neuropathological symptoms, a key feature that makes it an important model of HD. Changes between wild type and R6/2 mice can be seen as early as postnatal day 1, when huntingtin aggregates can first be detected in the neostriatum. However, most striking changes in neuropathology are not seen until later in the disease. Notable changes seen in the brain include an overall reduction in brain weight and volume, gross enlargement of lateral ventricles, neuronal atrophy, and presence of huntingtin aggregates[45]. These changes progress in severity over the course of the disease. Behavioral deficits, such as decreased performance on rotarod, correlate with histological markers such as the presence of huntingtin aggregates and changes in neuronal density[111]. Neuronal atrophy is a hallmark of the disease and can be quantified as a reduction in both size and number of neurons in the striatum[45]. In 12 week R6/2 mice, striatal neuronal count is reduced as much as 50% compared with wild type mice[112].

The pathology of HD is complex, and in addition to these known neurological characteristics, recent research highlighted the particular role apoptosis plays in neurodegeneration, indicating a possible mechanism for the damages caused by this disease[30,113,114]. Apoptosis, or programmed cell death, is characteristic of neurologic disease[115]. It is the primary form of cell death in neurodegenerative diseases, seen as a mechanism of neuron death in diseases such as Alzheimer's, stroke, and ALS[30].

Apoptosis results from a biochemical cascade. Early in this cascade, caspases are activated; these then eliminate molecules used to maintain cell survival[115]. The cascade causes cell structures condensation, the mitochondrial aggregation, and chromatin condensation. The cell fragments after its death and condensed chromatin is cleaved[30,115]. Apoptosis is the result of a complex interplay of molecular components.

Cysteine-dependent aspartate specific proteases (caspases) are primary factors in apoptosis. The caspase family is named for caspase 1, or interleukin-1β-converting enzyme[16]. Since its discovery, a total of 11 caspases have been identified in humans. The molecules begin as procaspases, which contain a recruitment domain at the N-terminal[16]. The type of recruitment domain helps indicate where in the apoptotic cascade a caspase functions. Caspases can function upstream or downstream within the cascade, initiating or executing, respectively. The N-terminal domain of most upstream molecules (procaspases 1, 2, 4, 5, 9, 11, 12, 13) is a caspase-recruiting domain (CARD). There are also two caspases (8 and 10) which have a death-effector domain (DED) at the N-terminus. These upstream caspases function to initiate the process of apoptosis. The other caspases (3, 6, 7, 14) have a short prodomain[30]. These caspases function in actual executing of the cell by both destroying cellular structures and degrading DNA[16]

In the R6/2 model of HD, the activation of caspases is known to play a critical role in disease progression with specific chronology of activation[35]. The activation of caspases 3 and 9 is noted to occur later in disease, while activation of caspase 1 occurs earlier[35,41]. Caspase 1, which acts upstream of the mitochondria, acts by cleaving Bid; the cleavage product (tBid) the localizes to the mitochondrial membrane where it effects the release of cytochrome *c*[35]. Cytochrome c initiates the caspase mediated apoptotic pathway downstream of the mitochondria. Previous work explored the ability of melatonin to inhibit cell death pathways; notably melatonin is able to inhibit

the activation of caspase 1 and caspase 3 and prevent cytochrome c release in both ALS and HD mouse models[65,100].

To further assess the role of MT1 in this melatonin mediated neuroprotection, I conducted experiments to determine whether overexpression of the receptor would further inhibit the activation of caspases and to assess neuronal atrophy via both neuronal count in wild type, R6/2, and NSE-MT1-R6/2 mice treated with saline or melatonin.

5.2 METHODS

Mice were treated and collected as described in section 3 of this dissertation. Mice tested for caspase activation consisted of wild type, R6/2, MT1, and NSE-MT1-R6/2 mice with both saline and melatonin treatment groups at 9 and 12 weeks of age. Neuronal counts were measured in control (wild type treated with saline) and R6/2 and NSE-MT1-R6/2 mice treated with saline and melatonin at 12 weeks of age, n=4 for each group.

5.2.1 Caspase activity assays

Previous work by the Friedlander group used fluorometric assays to assess caspase activity with high sensitivity[35]. Therefore, caspase activation in brains of these mice was also measured using fluorometric assay kits for caspases 1, 3, and 9 [Abcam, ab39412, ab39383, and ab65607, respectively]. These fluorometric kits used caspase specific substrates (YYAD-AFC, DEVD-AFC, and LEHD-AFC for 1, 3, and 9, respectively) which emit a blue light (400nm wavelength) when uncleaved and, when cleaved by their respective caspase free AFC (7-amino-4-

trifluoromethyl coumarin), which emits fluorescence (excitation 400nm, emission 505nm). Caspase activity is quantified by amount of free AFC produced.

While the kits were designed for use with cells, I adapted a protocol for use with mouse brain tissue from the manufacturer instructions, correspondence with manufacturer, and direct optimization: a half brain was homogenized in 700µL of kit lysis buffer and spun at 1000rpm for 5 minutes at 4°C to remove debris. From this, 500µg protein (total volume 50µL in lysis buffer) was combined with 50µL kit reaction buffer (containing 10mM DTT) and 5µL of 1mM substrate (caspase 1, 3, or 9 substrates, from kit). Plates of samples were incubated at 37°C for 2 hours and read on Synergy H1 with read settings described in kit protocol. Outputs were calculated as fold increase compared to control (wild type untreated sample).

5.2.2 Tissue collection and preparation

For neuropathology assessment, mice were treated and collected as described in section 4 of this dissertation. Mice tested consisted of control (wild type treated with saline) and R6/2 and NSE-MT1-R6/2 mice treated with saline and melatonin. For each measurement, a total of 4 mice were used.

Brains were collected at 12 weeks of age (late stage disease). Mice were cardiac perfused with approximately 60ml of 10% formalin. After perfusion, brains were collected and stored in 10% formalin at 4°C for 24 hours. Brains were then transferred to increasing concentrations of glycerol (10% followed by 20%). They were stored at 4°C in the final 20% glycerol solution until sectioned. Brains were coronally sectioned at 40µm thickness using a cryostat.

5.2.3 Histology

Sections were stained with cresyl violet (5 minutes xylenes, 5 minutes 100% ethanol, overnight 95% ethanol, 5 minutes 70% EtOH, 5 minutes ddH2O, 10 minutes cresyl violet, 5 minutes ddH2O, 5 minutes ddH2O, 5 minutes 70% EtOH, 5 minutes 95% EtOH, 5 minutes 95% EtOH, 5 minutes 100% EtOH, 30 sec 100% EtOH, 1 minutes xylenes) and analyzed for neuronal cell counts using Image-J software. For each mouse (n=4) sample, a total of 5 images were collected and the counts averaged.

5.2.4 Statistical analysis

Statistical analysis of caspase activation and neuronal counts was done using a 2-way ANOVA with Tukey's multiple comparisons test, generating multiplicity adjusted p-values for each cohort's comparison.

5.3 RESULTS

Caspase 1, 3, and 9 activity was assessed in age 9 weeks and 12 weeks mice using fluorometric assays and fold changes of activity were determined in comparison to untreated WT mice. For all caspases and timepoints, a significant difference can be seen between untreated WT and untreated R6/2, indicating the test is sensitive enough to detect the activation of these caspases (figure 9). With no significant difference for any timepoint of any caspase for untreated R6/2 vs untreated

NSE-MT1-R6/2, it can be seen that the addition of the MT1 transgenic receptor alone has no beneficial effect in these mice without the presence of melatonin.

At 9 weeks, an impact of treatment can only be seen with caspase 3 (figure 9c), where the decrease in activity is seen with the addition of treatment in the R6/2 genotype ($p=0.007$). At 12 weeks, impact of addition of melatonin to the R6/2 shows significant decreases in all three caspases (figure 9b, d, f). This is consistent with previous findings that show melatonin ameliorates disease phenotype, including caspase activation.

At 9 weeks, the treated NSE-MT1-R6/2 genotype appears to decrease caspase activation more strongly than treatment confers to R6/2 genotype alone (figure 9a, c, e), although this trend is not significant at the 9 week timepoint.

However, at 12 weeks, this difference becomes significant for caspases 3 and 9 ($p<0.0001$ and $p=0.011$, respectively) (figure 9d and f), indicating that addition of MT1 strengthens the protective effects of melatonin treatment. However, this improvement appears very late in the disease (near the end of lifespan for the R6/2 model), and it appears not to produce a strong enough effect to see major changes on behavior or survival.

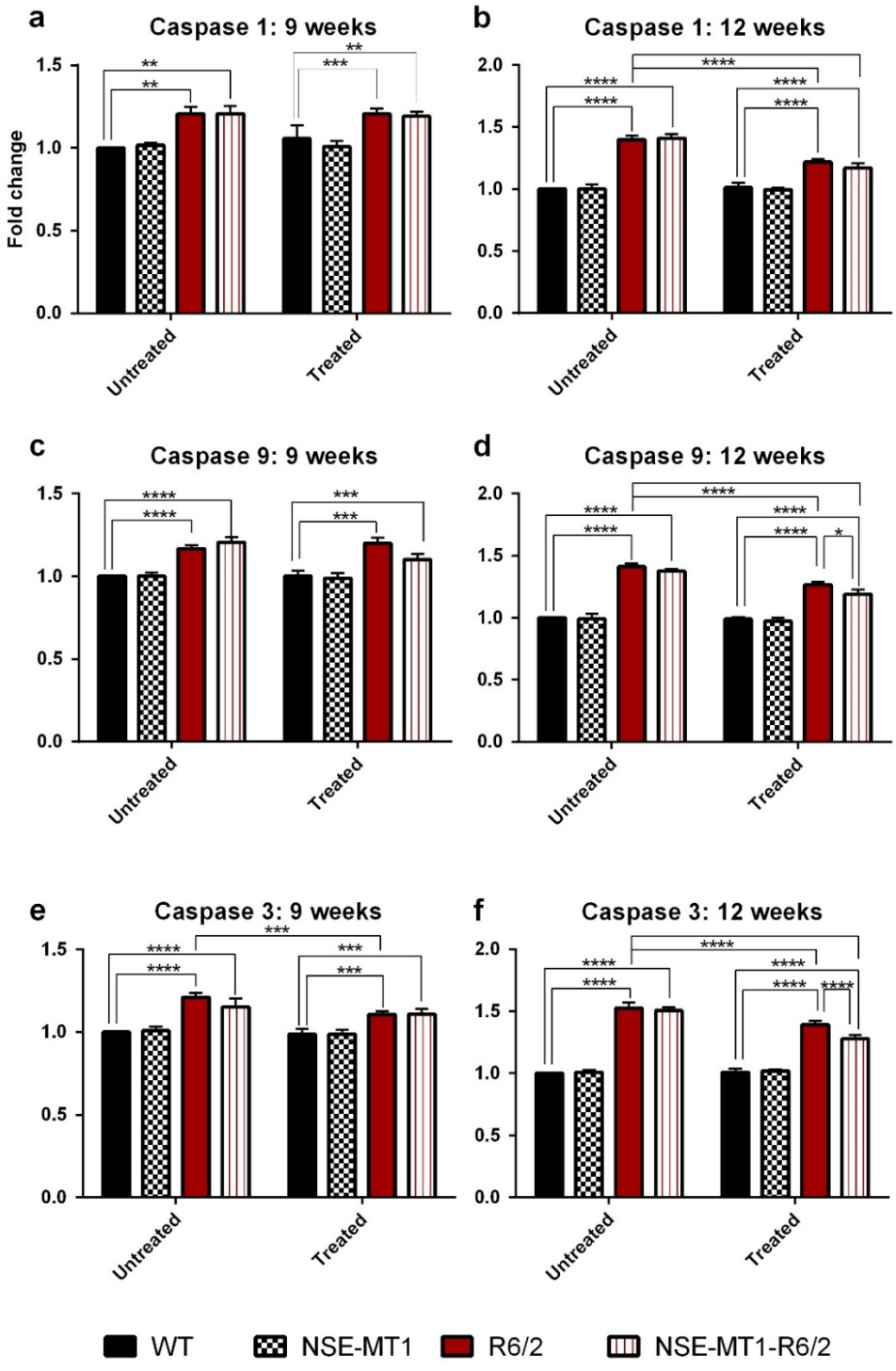

Figure 9. Caspase 1, 9, and 3 activity in WT, NSE-MT1, R6/2, and NSE-MT1-R6/2 mice, treated with saline or melatonin.

Caspase activation in whole brain homogenate was determined using fluorogenic assays. Activation is shown as fold change from saline treated wild type. For each sample group, n=3 with individual samples run in triplicate. Activation was assessed at mid-stage (9 weeks) and late-stage (12 weeks) of disease. Notable significance is indicated with asterisks (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).

Table 2. Summary of p values for all comparisons of caspase activation between genotypes and treatments at 9 and 12 weeks of age.

				saline			melatonin			
				NSE-MT1	R6/2	NSE-MT1-R6/2	WT	NSE-MT1	R6/2	NSE-MT1-R6/2
Caspase 1	9 weeks	saline	WT	0.9991	<0.0001	<0.0001	0.5385	>0.9999	<0.0001	<0.0001
			NSE-MT1		<0.0001	<0.0001	0.8563	>0.9999	<0.0001	<0.0001
			R6/2			>0.9999	0.0006	<0.0001	>0.9999	0.9993
			NSE-MT1-R6/2				0.0006	<0.0001	>0.9999	0.9995
		melatonin	WT					0.7177	0.0006	0.0023
			NSE-MT1						<0.0001	<0.0001
			R6/2							0.9994
	12 weeks	saline	WT	>0.9999	<0.0001	<0.0001	0.9984	>0.9999	<0.0001	<0.0001
			NSE-MT1		<0.0001	<0.0001	0.9990	>0.9999	<0.0001	<0.0001
			R6/2			0.9990	<0.0001	<0.0001	<0.0001	<0.0001
			NSE-MT1-R6/2				<0.0001	<0.0001	<0.0001	<0.0001
		melatonin	WT					0.9886	<0.0001	<0.0001
			NSE-MT1						<0.0001	<0.0001
			R6/2							04312
Caspase 9	9 weeks	saline	WT	>0.9999	<0.0001	<0.0001	>0.9999	0.9988	<0.0001	0.0006
			NSE-MT1		<0.0001	<0.0001	>0.9999	.9963	<0.0001	0.0008
			R6/2			0.5896	<0.0001	<0.0001	0.7777	0.0486
			NSE-MT1-R6/2				<0.0001	<0.0001	>0.9999	0.0006
		melatonin	WT					0.9971	<0.0001	0.0007
			NSE-MT1						<0.0001	0.0001
			R6/2							0.0013
	12 weeks	saline	WT	0.9997	<0.0001	<0.0001	0.9997	0.8424	<0.0001	<0.0001
			NSE-MT1		<0.0001	<0.0001	>0.9999	0.9794	<0.0001	<0.0001
			R6/2			0.6084	<0.0001	<0.0001	<0.0001	<0.0001
			NSE-MT1-R6/2				<0.0001	<0.0001	0.0001	<0.0001
		melatonin	WT					0.9777	<0.0001	<0.0001
			NSE-MT1						<0.0001	<0.0001
			R6/2							0.011
Caspase 3	9 weeks	saline	WT	0.9998	<0.0001	<0.0001	0.9988	0.9977	0.0005	0.0004
			NSE-MT1		<0.0001	<0.0001	0.9636	0.9511	0.0016	0.0013
			R6/2			0.1190	<0.0001	<0.0001	0.0007	0.0009
			NSE-MT1-R6/2				<0.0001	<0.0001	0.3817	0.4294
		melatonin	WT					>0.9999	0.0001	0.0001
			NSE-MT1						0.0001	<0.0001
			R6/2							>0.9999
	12 weeks	saline	WT	0.9994	<0.0001	<0.0001	0.9995	0.9462	<0.0001	<0.0001
			NSE-MT1		<0.0001	<0.0001	>0.9999	0.9986	<0.0001	<0.0001
			R6/2			0.9722	<0.0001	<0.0001	<0.0001	<0.0001
			NSE-MT1-R6/2				<0.0001	<0.0001	<0.0001	<0.0001
		melatonin	WT					0.9983	<0.0001	<0.0001
			NSE-MT1						<0.0001	<0.0001
			R6/2							<0.0001

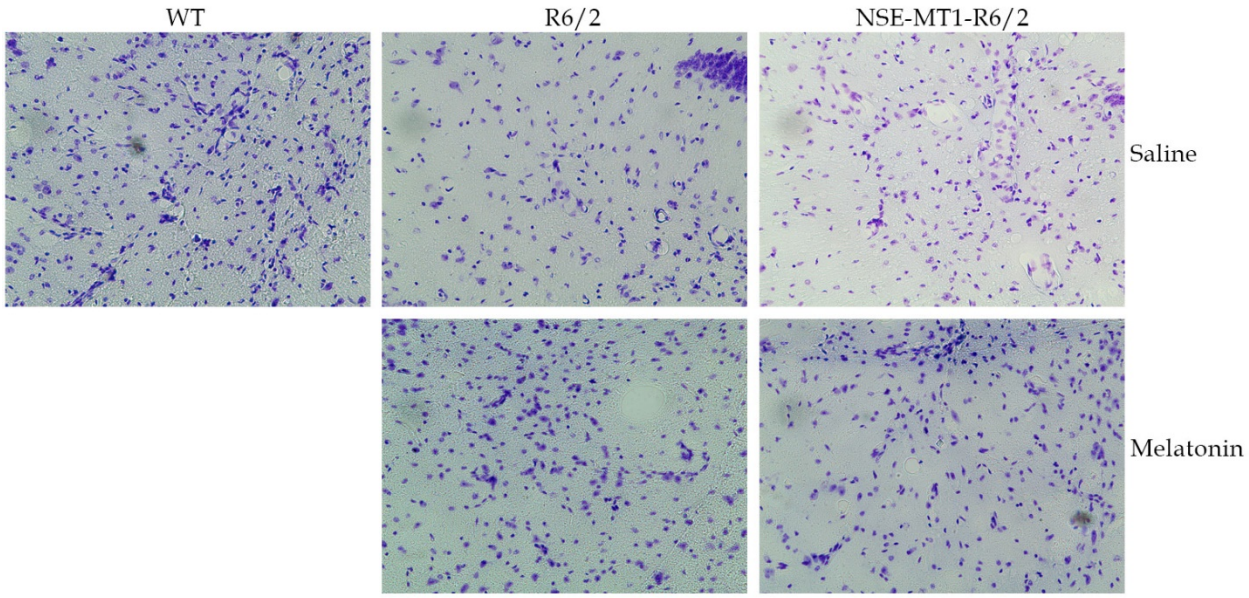

Figure 10. Representative images of neuronal density and number in striatum of WT, R6/2, and NSE-MT1-R6/2 mice, treated with saline or melatonin.
Neuronal counts, an indication of neuronal cell death, were assessed in striatum of 30μm brain sections stained with cresyl violet (Nissl stain).

As expected, neuronal number was significantly decreased in the striatum of saline treated mice with the R6/2 genotype compared to WT, with the number reduced by 42.7% in R6/2 compared with WT ($p=0.0007$) and 37.6% in NSE-MT1-R6/2 compared with WT ($p=0.0019$). This reduction was ameliorated by treatment with melatonin. In the NSE-MT1-R6/2, treatment with melatonin significantly increased neuronal number by 27.2% ($p=0.0442$). While there were consistently higher counts in the NSE-MT1-R6/2 melatonin treatment compared to R6/2 melatonin treatment, the perceived difference was not reach significant ($p=0.2889$).

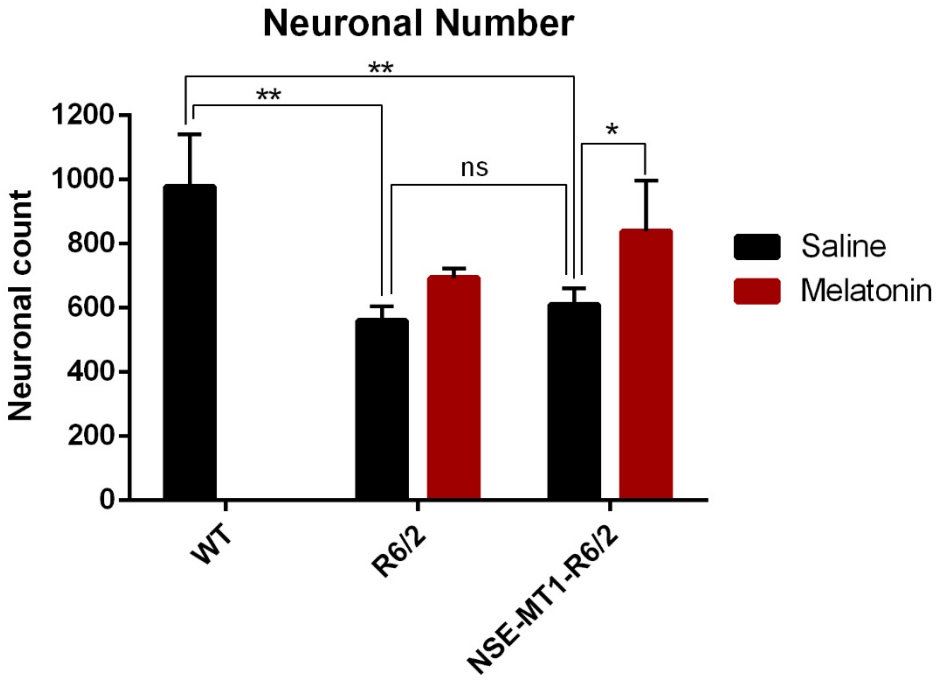

Figure 11. Neuronal counts from WT, R6/2, and NSE-MT1-R6/2 treated with saline or melatonin. Neuronal number in each section was analyzed with ImageJ. Quantification is the average of 5 image sections per brain, n=4 brains per genotype/treatment. Significance is indicated with asterisks (ns: no significance, *: $p<0.05$,**: $p<0.01$).

5.4 DISCUSSION

While the NSE-MT1-R6/2 genotype did not show significant improvement in rotarod performance or survival, significant changes in caspase activation were detected. While it is possible the R6/2 phenotype is too aggressive to see macro changes at the level of behavior and survival, I determined to assess whether the differences in caspase activation were able to still make subtle changes at the neuronal level.

Treatment with melatonin resulted in significant reduction of caspase activation in the diseased mice. This finding is consistent with previous work indicating that melatonin is able to reduce neuronal cell death. The ability of melatonin to reduce caspase activity, and for this effect

to be strengthened by the addition of the MT1 transgenic, illustrates that MT1 does play an important role in mediating melatonin neuroprotection. Similar to the results seen with behavior and survival, this effect shows up very late in the disease and appears not to provide a strong enough biological impact to prolong survival or alter disease course. This data, in combination with the outcomes seen with behavior and survival, provides a more complete picture of the roles of MT1 in melatonin neuroprotection, although there are still multiple potential conclusions to be explored further.

The inability of this caspase activity rescue to significantly impact the disease course at a macro level (i.e. behavior and survival) may be in part due to the aggressive nature of the R6/2 HD phenotype, as mice begin dying before a significant effect takes place. The R6/2 model of HD is indeed one of the fastest and most aggressive models available to study the disease. While the model has the advantage of also being among the most consistent in terms of its onset and progression timeline, this also results in an early death. While the protective effect of melatonin itself can be clearly seen within the lifespan of the mouse, amelioration due to the addition of the MT1 overexpression may not be additionally strong enough to elicit an outcome within the mouse's lifespan. A clearer result may theoretically be possible by using a slower progressing model or a greater overexpression of MT1. The latter option seems less feasible as our higher expressing model of the MT1 transgenic (the 2606 line) displays significant issues in breeding. Due to time constraints and mouse availability, experimentation with an alternate HD model mouse or the higher expressing MT1 line were not pursued for this project, but remain a potential path for future study.

In addition to the caspase activation data, neuronal counts provide key insight into the subtle changes the NSE-MT1-R6/2 genotype provided when combined with melatonin treatment.

The changes at the cellular level shown here follow the pattern of changes seen with caspase activation and rescue, although with a milder effect. This slight perceived difference continues to be seen at the behavioral level, with the effect again being even more mild (non-significant). The neuropathological data provides a connecting line between understanding the role of neuroprotection against apoptotic factors and an overall alteration in behavior and survival. However, the MT1 overexpression ultimately did not provide a strong additive effect to the administration of melatonin alone.

6.0 CONCLUSIONS & DISCUSSION

HD is a devastating disease with no known cure or treatment. Finding mechanisms of its pathology as well as mechanisms of protection are critically important to helping individuals and families with this disease.

Melatonin has been widely studied in a variety of roles, including in circadian rhythms, as an antioxidant, and most recently as a neuroprotectant. While melatonin is known to improve outcomes for other neurodegenerative diseases such as Parkinson's and Alzheimer's, its ability to ameliorate symptoms in HD is a promising new development. In order to fully investigate the neuroprotective effects of melatonin, we must develop a clear picture of the mechanisms of melatonin's function within cells. Because melatonin functions in cells through multiple mechanisms, including both antioxidant and receptor mediated roles, understanding how these functions contribute to its neuroprotective properties allows us to direct efforts to maximize neuroprotection as we aim to develop treatments for individuals with HD.

Melatonin signaling occurs via two receptors, MT1 and MT2. Because the neuroprotective effects of melatonin occur through MT1, it is the focus of this research. The Friedlander laboratory previously found MT1 is located on the mitochondria. Melatonin's highest concentration within the cell is also the mitochondria, although the mechanism of melatonin's mitochondrial localization was unclear. The Friedlander group identified the two terminal enzymes involved in melatonin synthesis, AANAT and ASMT, as being localized to the mitochondria, pointing to the possibility of their function being carried out there. Here, I demonstrate isolated mitochondria are capable of synthesizing melatonin from serotonin. This centralizes mitochondria in understanding the mechanisms of melatonin's neuroprotection via MT1. This also establishes a novel autocrine

loop for the synthesis and signaling of neuronal melatonin. Melatonin is synthesized in mitochondria, where is binds to its MT receptors. Melatonin does not need to be transported within the cell or from external sources in order for rapid signaling to occur. No other known autocrine signaling in the mitochondria is documented in literature. Ongoing studies are aimed at examining how this melatonin synthesis in the mitochondria may be affected by the HD phenotype, using the R6/2 mouse model.

The relationship between mitochondria and HD is well documented. While there are many theories as to the precise role the mitochondria plays in HD pathology, it is clear that many functions of the mitochondria, from protein import to caspase mediated cell death to antioxidation, are affected in HD. Knowing melatonin synthesis and melatonin's signaling via MT1 both occur in the mitochondria, I sought to determine whether MT1 may also be affected by HD. Previous work with an R6/2 model with longer CAG repeat length showed MT1 expression decreased. My experiments sought to determine whether this downregulation occurred in the standard R6/2 mouse model, with approximately 144 CAG repeats. Indeed MT1 is downregulated in this R6/2 line at both an mRNA and protein level, seen at mid-stage disease and progressing to a larger significant difference by late stage disease. Not only does the HD phenotype decrease MT1 decrease but it does so in a progressive fashion correlating with disease progression. As previous research indicated MT1 as the mechanism of melatonin's neuroprotection, this decrease suggests the downregulation of MT1 contributes to increased neuronal death as the disease progresses.

The NSE-MT1 mouse model was developed to explore the effects of increased MT1 signaling. This increase can ameliorate the effects of decreased MT1 expression in an HD model such as the R6/2 and increase the efficacy of neuroprotection with melatonin. R6/2 and NSE-MT1 mice were crossed to create NSE-MT1-R6/2 offspring. I tested whether the decrease in

endogenous MT1 expression was paralleled with transgenic MT1. While the mechanism by which R6/2 downregulates endogenous MT1 is unknown, it may act through mechanisms such as transcriptional regulation, DNA modification, or mRNA degradation. These mechanisms could potentially affect the transgenic MT1, preventing an effective increase in MT1 expression in the NSE-MT1-R6/2 model. I found transgenic MT1 mRNA expression was the same with and without the addition of the R6/2 genotype at late stage disease (12 weeks). This provided a solid rationale for using the cross mice for experiments with melatonin neuroprotection; the increased transgenic MT1 compensates for the loss of endogenous MT1 in the R6/2 model without itself being reduced by the disease phenotype. Additionally, with the transgenic unaffected but endogenous decreased in R6/2, possible mechanisms of the latter can be posited. NSE-MT1 contains the human MT1 gene; while similar and homologous to the mouse MT1, it is not identical. While a search of the database GEO found no known microRNAs which target MT1 and are upregulated in R6/2 mice, factors such as changes to transcriptional regulators may affect endogenous mouse MT1 in a site specific way. Additionally, and potentially more likely an explanation, the endogenous and transgenic MT1 have different promoters, which may play a role in how the HD phenotype affects each due to HD affecting the endogenous MT1 promoter via changes in enhancers or transcriptional regulators. Mutant HTT is known to interfere with transcription factors and transcriptional proteins, cause histone and methylation based regulation of DNA, and alter miRNA expression. The results of these experiments opens a new understanding of how the HD phenotype, as seen in the R6/2, may affect melatonin signaling via MT1. In a broader scheme, insight into how HD downregulates MT1 expression may be useful in understanding HD's many gene regulation effects.

Because the transgenic MT1 is not affected by the R6/2 genotype, it has the potential to rescue the loss of melatonin signaling via MT1 and increase the neuroprotective effects of melatonin. The R6/2 and NSE-MT1 mice were crossed to investigate the capability of increased MT1 expression to improve melatonin's previously seen amelioration of the disease phenotype.

At a molecular level, melatonin is able to inhibit cell death via caspase-mediated pathways, although the specific molecular mechanism responsible is unknown. The overexpression of MT1 produced a modest but significant improvement on reduction of caspase activation, particularly at late-stage disease. This demonstrates that a potential pathway for melatonin signaling via MT1 to prevent activation of cell death pathways. However, further experiments to examine the effects of this at a macro level were less conclusive. While cell death via caspase-mediated pathways appears decreased, cell death may still be occurring via other pathways. In order to assess whether this reduction in caspase activation was truly protective from cell death, an assessment of neuronal death was needed. Neuronal counts suggested improvement with the addition of the transgenic MT1 but were still statistically decreased compared to controls. Similarly, the measures of disease onset and progression using rotarod testing suggested an improved phenotype in the NSE-MT1-R6/2 compared to R6/2 alone, however these rotarod measures only conclusively demonstrated improved at very late stage disease. As with other endpoint measures, data suggested but could not conclusively demonstrate improved survival. While caspase activation was further decreased in melatonin treated NSE-MT1-R6/2 compared to R6/2 alone, reducing caspase-mediated cell death, the data do not suggest this decrease is sufficient to decrease neuronal cell death and ameliorate the disease phenotype. There are several possible explanations for this outcome including choice of mouse models, feedback mechanisms, modification of MT1 signaling functions, alternative pathways of neuroprotection, or insufficient melatonin dosing.

With improved outcomes seen primarily in late stage disease, there is the possibility that the mice die before a strong and significant effect of the neuroprotection can be seen. While the R6/2 model is advantageous for its robust disease phenotype and clear progressive pathology, it also has the disadvantage of being a particularly aggressive and quick model. As a result, the effects of MT1 overexpression may have been more difficult to see at a macro phenotypic level. The use of a slower disease model in future work may allow for seeing a stronger effect of MT1 overexpression.

Also factoring in to the efficacy of the mouse models is the possibility that transgenic MT1 expression may not have been high enough. Of the two founder lines for the NSE-MT1 mouse model, the line used for this work, line 2604, had lower expression of the transgenic MT1 compared to the second founder line 2606. Future work in the understanding of increased melatonin signaling via MT1 may require use of the higher expressing line with the aggressive R6/2 phenotype. Additionally, research exploring melatonin's neuroprotection as it functions through MT1 would benefit from pairing both overexpressed MT1 models and knockout MT1 models with HD mouse modeling. Given the slight improvements in disease phenotype with the addition of excess MT1, one would expect to see all melatonin-related neuroprotection eliminated with the knockout of MT1.

Conversely, it is possible that transgenic MT1 expression was high, but the melatonin dose was not optimal. While the melatonin dosage was optimized to be a saturating level in the R6/2 mouse, the dose given may not have been saturating for the increased amount of MT1. In future studies, dosing should be determined for the overexpressed MT1 levels, allowing for a level of melatonin which would fully saturate the transgenic MT1 and maximize increased signaling via MT1. Alternatively, no lower doses were tested during optimization and it is possible the dose of

melatonin is sufficient to result in saturating downstream signaling, such that an increased number of receptors would not affect the already saturated signaling pathway.

The expression of endogenous MT1 may be affected by transgenic MT1 expression via a regulatory feedback mechanism. If there is a maximal level of MT1 expression in a cell, the transgenic expression may cause a reduction in endogenous MT1 in order to maintain this level, ultimately resulting in the same final amount of MT1 expressed.

Additionally, it is important to note the dimerization of MT1 with itself, and as a heterodimer with MT2 or GPR50. These are of particular interest due to their ability to reduce MT1 function when dimerized, with GPR50 providing the most striking reduction in function. While research is still determining the function of GPR50, some hypotheses point to it having a regulatory role in melatonin signaling. In this case, GPR50 may modify the functionality of the transgenic MT1 without resulting in a reduction of MT1 expression; this would account for a mild but not strong effect of MT1 overexpression. Future studies may benefit from knocking down GPR50 expression to determine whether a reduction in its ability to dimerize with MT1 results in stronger transgenic MT1 functionality and protective effects.

In addition to receptor changes, another factor affecting these outcomes is melatonin's role as an antioxidant. The HD phenotype has many molecular effects including both apoptotic dysfunction and oxidative damage. While these experiments focused on melatonin's role in inhibiting apoptosis, it may also provide neuroprotection as an antioxidant. Reactive oxygen species (ROS) are formed as a by-product of oxidative phosphorylation, the process by which ATP is produced in the mitochondria. Build-up of ROS in cells causes mitochondria damage, which is detected by BCL-2 family proteins on the mitochondria, triggering apoptotic cell death. In HD, there is an overproduction of ROS, increasing mitochondria and cell damage[116]. Melatonin

functions as an antioxidant, and it may protect against neuronal death by reducing oxidative damage in mitochondria and preventing apoptotic cell death in that manner.

The caspase activation changes paired with the neuropathological and behavioral phenotypic outcomes in the NSE-MT1-R6/2 compared to R6/2 alone increase our understanding of the strengths and limitations of melatonin-mediated neuroprotection. This provides key insight into MT1-mediated neuroprotection, an important mechanism for modifying the disease outcome. The data strengthens evidence for caspase reduction as a means of neuroprotection. However, there are notable limitations with the aggressive nature of the disease as modeled in R6/2 mice as well as the variety of unknown co-regulators which may impact the outcomes beyond MT1's effects alone. Combined with research in the field of understanding the pathology of HD and development of melatonergic drugs, my work provides a foundation for better understanding how to treat and modify this fatal disease.

BIBLIOGRAPHY

1 Huntington, G. On chorea. *The Journal of neuropsychiatry and clinical neurosciences* **26**, 317-321 (1872).

2 Gilliam, T. C. *et al.* Localization of the Huntington's disease gene to a small segment of chromosome 4 flanked by D4S10 and the telomere. *Cell* **50**, 565-571 (1987).

3 A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell* **72**, 971-983 (1993).

4 Ambrose, C. M. *et al.* Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. *Somatic cell and molecular genetics* **20**, 27-38 (1994).

5 Pringsheim, T. *et al.* The incidence and prevalence of Huntington's disease: a systematic review and meta-analysis. *Movement disorders : official journal of the Movement Disorder Society* **27**, 1083-1091, doi:10.1002/mds.25075 (2012).

6 Semaka, A., Creighton, S., Warby, S. & Hayden, M. R. Predictive testing for Huntington disease: interpretation and significance of intermediate alleles. *Clinical genetics* **70**, 283-294, doi:10.1111/j.1399-0004.2006.00668.x (2006).

7 Andrew, S. E. *et al.* The relationship between trinucleotide (CAG) repeat length and clinical features of Huntington's disease. *Nature genetics* **4**, 398-403, doi:10.1038/ng0893-398 (1993).

8 Brandt, J. *et al.* Trinucleotide repeat length and clinical progression in Huntington's disease. *Neurology* **46**, 527-531 (1996).

9 Claes, S. *et al.* Correlations between triplet repeat expansion and clinical features in Huntington's disease. *Archives of neurology* **52**, 749-753 (1995).

10 Stine, O. C. *et al.* Correlation between the onset age of Huntington's disease and length of the trinucleotide repeat in IT-15. *Human molecular genetics* **2**, 1547-1549 (1993).

11 Wexler, N. S. *et al.* Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington's disease age of onset. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3498-3503, doi:10.1073/pnas.0308679101 (2004).

12 Li, J. L. *et al.* Genome-wide significance for a modifier of age at neurological onset in Huntington's disease at 6q23-24: the HD MAPS study. *BMC medical genetics* **7**, 71, doi:10.1186/1471-2350-7-71 (2006).

13 Kremer, B. *et al.* Sex-dependent mechanisms for expansions and contractions of the CAG repeat on affected Huntington disease chromosomes. *American journal of human genetics* **57**, 343-350 (1995).

14 Telenius, H. *et al.* Somatic and gonadal mosaicism of the Huntington disease gene CAG repeat in brain and sperm. *Nature genetics* **6**, 409-414, doi:10.1038/ng0494-409 (1994).

15 Gonzalez-Alegre, P. & Afifi, A. K. Clinical characteristics of childhood-onset (juvenile) Huntington disease: report of 12 patients and review of the literature. *Journal of child neurology* **21**, 223-229 (2006).

16 Sanchez Mejia, R. O. & Friedlander, R. M. Caspases in Huntington's disease. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* **7**, 480-489 (2001).

17 Walker, F. O. Huntington's disease. *Lancet* **369**, 218-228, doi:10.1016/S0140-6736(07)60111-1 (2007).

18 Snowden, J. S., Craufurd, D., Griffiths, H. L. & Neary, D. Awareness of involuntary movements in Huntington disease. *Archives of neurology* **55**, 801-805 (1998).

19 Anderson, K. E. & Marder, K. S. An overview of psychiatric symptoms in Huntington's disease. *Current psychiatry reports* **3**, 379-388 (2001).

20 Kirkwood, S. C., Su, J. L., Conneally, P. & Foroud, T. Progression of symptoms in the early and middle stages of Huntington disease. *Archives of neurology* **58**, 273-278 (2001).

21 Mahant, N., McCusker, E. A., Byth, K., Graham, S. & Huntington Study, G. Huntington's disease: clinical correlates of disability and progression. *Neurology* **61**, 1085-1092 (2003).

22 Aziz, N. A. *et al.* Weight loss in Huntington disease increases with higher CAG repeat number. *Neurology* **71**, 1506-1513, doi:10.1212/01.wnl.0000334276.09729.0e (2008).

23 Djousse, L. *et al.* Weight loss in early stage of Huntington's disease. *Neurology* **59**, 1325-1330 (2002).

24 Marder, K. *et al.* Dietary intake in adults at risk for Huntington disease: analysis of PHAROS research participants. *Neurology* **73**, 385-392, doi:10.1212/WNL.0b013e3181b04aa2 (2009).

25 Robbins, A. O., Ho, A. K. & Barker, R. A. Weight changes in Huntington's disease. *European journal of neurology : the official journal of the European Federation of Neurological Societies* **13**, e7, doi:10.1111/j.1468-1331.2006.01319.x (2006).

26 Sorensen, S. A. & Fenger, K. Causes of death in patients with Huntington's disease and in unaffected first degree relatives. *Journal of medical genetics* **29**, 911-914 (1992).

27 Vonsattel, J. P. *et al.* Neuropathological classification of Huntington's disease. *Journal of neuropathology and experimental neurology* **44**, 559-577 (1985).

28 Shoulson, I. & Young, A. B. Milestones in huntington disease. *Movement disorders : official journal of the Movement Disorder Society* **26**, 1127-1133, doi:10.1002/mds.23685 (2011).

29 Mitchell, I. J., Cooper, A. J. & Griffiths, M. R. The selective vulnerability of striatopallidal neurons. *Progress in neurobiology* **59**, 691-719 (1999).

30 Friedlander, R. M. Apoptosis and caspases in neurodegenerative diseases. *The New England journal of medicine* **348**, 1365-1375, doi:10.1056/NEJMra022366 (2003).

31 Galluzzi, L. *et al.* Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell death and differentiation* **19**, 107-120, doi:10.1038/cdd.2011.96 (2012).

32 Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D. & Korsmeyer, S. J. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**, 334-336, doi:10.1038/348334a0 (1990).

33 Gross, A., McDonnell, J. M. & Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. *Genes & development* **13**, 1899-1911 (1999).

34 Li, H., Zhu, H., Xu, C. J. & Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491-501 (1998).

35 Zhang, Y. *et al.* Sequential activation of individual caspases, and of alterations in Bcl-2 proapoptotic signals in a mouse model of Huntington's disease. *Journal of neurochemistry* **87**, 1184-1192 (2003).

36 Li, P. *et al.* Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479-489 (1997).

37 Kuida, K. *et al.* Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94**, 325-337 (1998).

38 Radi, E., Formichi, P., Battisti, C. & Federico, A. Apoptosis and oxidative stress in neurodegenerative diseases. *Journal of Alzheimer's disease : JAD* **42 Suppl 3**, S125-152, doi:10.3233/JAD-132738 (2014).

39 Ona, V. O. *et al.* Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. *Nature* **399**, 263-267, doi:10.1038/20446 (1999).

40 Chen, M. *et al.* Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nature medicine* **6**, 797-801, doi:10.1038/77528 (2000).

41 Kiechle, T. *et al.* Cytochrome C and caspase-9 expression in Huntington's disease. *Neuromolecular medicine* **1**, 183-195, doi:10.1385/NMM:1:3:183 (2002).

42 Menalled, L. B. & Chesselet, M. F. Mouse models of Huntington's disease. *Trends in pharmacological sciences* **23**, 32-39 (2002).

43 Menalled, L. B. *et al.* Comprehensive behavioral and molecular characterization of a new knock-in mouse model of Huntington's disease: zQ175. *PloS one* **7**, e49838, doi:10.1371/journal.pone.0049838 (2012).

44 Brooks, S. P. & Dunnett, S. B. Mouse Models of Huntington's Disease. *Current topics in behavioral neurosciences*, doi:10.1007/7854_2013_256 (2013).

45 Stack, E. C. *et al.* Chronology of behavioral symptoms and neuropathological sequela in R6/2 Huntington's disease transgenic mice. *The Journal of comparative neurology* **490**, 354-370, doi:10.1002/cne.20680 (2005).

46 Li, J. Y., Popovic, N. & Brundin, P. The use of the R6 transgenic mouse models of Huntington's disease in attempts to develop novel therapeutic strategies. *NeuroRx : the*

journal of the American Society for Experimental NeuroTherapeutics **2**, 447-464, doi:10.1602/neurorx.2.3.447 (2005).

47 Mangiarini, L. *et al.* Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* **87**, 493-506 (1996).

48 Luesse, H. G. *et al.* Evaluation of R6/2 HD transgenic mice for therapeutic studies in Huntington's disease: behavioral testing and impact of diabetes mellitus. *Behavioural brain research* **126**, 185-195 (2001).

49 Mihm, M. J. *et al.* Cardiac dysfunction in the R6/2 mouse model of Huntington's disease. *Neurobiology of disease* **25**, 297-308, doi:10.1016/j.nbd.2006.09.016 (2007).

50 Lerner, A. B., Case, J. D. & Takahashi, Y. Isolation of melatonin and 5-methoxyindole-3-acetic acid from bovine pineal glands. *The Journal of biological chemistry* **235**, 1992-1997 (1960).

51 Lynch, H. J., Wurtman, R. J., Moskowitz, M. A., Archer, M. C. & Ho, M. H. Daily rhythm in human urinary melatonin. *Science* **187**, 169-171 (1975).

52 Poeggeler, B., Reiter, R. J., Tan, D. X., Chen, L. D. & Manchester, L. C. Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. *Journal of pineal research* **14**, 151-168 (1993).

53 Reiter, R. J. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. *Endocrine reviews* **12**, 151-180, doi:10.1210/edrv-12-2-151 (1991).

54 Venegas, C. *et al.* Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations. *Journal of pineal research* **52**, 217-227, doi:10.1111/j.1600-079X.2011.00931.x (2012).

55 Reiter, R. J. & Tan, D. X. Role of CSF in the transport of melatonin. *Journal of pineal research* **33**, 61 (2002).

56 Ozaki, Y. & Lynch, H. J. Presence of melatonin in plasma and urine or pinealectomized rats. *Endocrinology* **99**, 641-644 (1976).

57 Acuna-Castroviejo, D. *et al.* Extrapineal melatonin: sources, regulation, and potential functions. *Cellular and molecular life sciences : CMLS* **71**, 2997-3025, doi:10.1007/s00018-014-1579-2 (2014).

58 Waldhauser, F. *et al.* Alterations in nocturnal serum melatonin levels in humans with growth and aging. *The Journal of clinical endocrinology and metabolism* **66**, 648-652, doi:10.1210/jcem-66-3-648 (1988).

59 Aziz, N. A. *et al.* Delayed onset of the diurnal melatonin rise in patients with Huntington's disease. *Journal of neurology* **256**, 1961-1965, doi:10.1007/s00415-009-5196-1 (2009).

60 Kalliolia, E. *et al.* Plasma melatonin is reduced in Huntington's disease. *Movement disorders : official journal of the Movement Disorder Society* **29**, 1511-1515, doi:10.1002/mds.26003 (2014).

61 Goodman, A. O., Morton, A. J. & Barker, R. A. Identifying sleep disturbances in Huntington's disease using a simple disease-focused questionnaire. *PLoS currents* **2**, RRN1189, doi:10.1371/currents.RRN1189 (2010).

62 Vivien-Roels, B. *et al.* Daily variations in pineal melatonin concentrations in inbred and outbred mice. *Journal of biological rhythms* **13**, 403-409 (1998).

63 Kasahara, T., Abe, K., Mekada, K., Yoshiki, A. & Kato, T. Genetic variation of melatonin productivity in laboratory mice under domestication. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 6412-6417, doi:10.1073/pnas.0914399107 (2010).

64 Morton, A. J. *et al.* Disintegration of the sleep-wake cycle and circadian timing in Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**, 157-163, doi:10.1523/JNEUROSCI.3842-04.2005 (2005).

65 Wang, X. *et al.* The melatonin MT1 receptor axis modulates mutant Huntingtin-mediated toxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**, 14496-14507, doi:10.1523/JNEUROSCI.3059-11.2011 (2011).

66 Slaugenhaupt, S. A. *et al.* Mapping of the gene for the Mel1a-melatonin receptor to human chromosome 4 (MTNR1A) and mouse chromosome 8 (Mtnr1a). *Genomics* **27**, 355-357, doi:10.1006/geno.1995.1056 (1995).

67 Roca, A. L., Godson, C., Weaver, D. R. & Reppert, S. M. Structure, characterization, and expression of the gene encoding the mouse Mel1a melatonin receptor. *Endocrinology* **137**, 3469-3477, doi:10.1210/endo.137.8.8754776 (1996).

68 Dubocovich, M. L. & Markowska, M. Functional MT1 and MT2 melatonin receptors in mammals. *Endocrine* **27**, 101-110, doi:10.1385/ENDO:27:2:101 (2005).

69 Brydon, L. *et al.* Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins. *Molecular endocrinology* **13**, 2025-2038, doi:10.1210/mend.13.12.0390 (1999).

70 Morgan, P. J., Barrett, P., Howell, H. E. & Helliwell, R. Melatonin receptors: localization, molecular pharmacology and physiological significance. *Neurochemistry international* **24**, 101-146 (1994).

71 McNulty, S., Ross, A. W., Barrett, P., Hastings, M. H. & Morgan, P. J. Melatonin regulates the phosphorylation of CREB in ovine pars tuberalis. *Journal of neuroendocrinology* **6**, 523-532 (1994).

72 Bouvier, M. Oligomerization of G-protein-coupled transmitter receptors. *Nature reviews. Neuroscience* **2**, 274-286, doi:10.1038/35067575 (2001).

73 Reppert, S. M., Weaver, D. R., Ebisawa, T., Mahle, C. D. & Kolakowski, L. F., Jr. Cloning of a melatonin-related receptor from human pituitary. *FEBS letters* **386**, 219-224 (1996).

74 Li, D. Y. *et al.* Melatonin receptor genes in vertebrates. *International journal of molecular sciences* **14**, 11208-11223, doi:10.3390/ijms140611208 (2013).

75 Grunewald, E., Kinnell, H. L., Porteous, D. J. & Thomson, P. A. GPR50 interacts with neuronal NOGO-A and affects neurite outgrowth. *Molecular and cellular neurosciences* **42**, 363-371, doi:10.1016/j.mcn.2009.08.007 (2009).

76 Levoye, A. *et al.* The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *The EMBO journal* **25**, 3012-3023, doi:10.1038/sj.emboj.7601193 (2006).

77 Wu, Y. H., Zhou, J. N., Van Heerikhuize, J., Jockers, R. & Swaab, D. F. Decreased MT1 melatonin receptor expression in the suprachiasmatic nucleus in aging and Alzheimer's disease. *Neurobiology of aging* **28**, 1239-1247, doi:10.1016/j.neurobiolaging.2006.06.002 (2007).

78 Adi, N. *et al.* Melatonin MT1 and MT2 receptor expression in Parkinson's disease. *Medical science monitor : international medical journal of experimental and clinical research* **16**, BR61-67 (2010).

79 Sanchez-Hidalgo, M. *et al.* Age-related changes in melatonin synthesis in rat extrapineal tissues. *Experimental gerontology* **44**, 328-334, doi:10.1016/j.exger.2009.02.002 (2009).

80 Uz, T. *et al.* The regional and cellular expression profile of the melatonin receptor MT1 in the central dopaminergic system. *Brain research. Molecular brain research* **136**, 45-53, doi:10.1016/j.molbrainres.2005.01.002 (2005).

81 Dubocovich, M. L. *et al.* International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. *Pharmacological reviews* **62**, 343-380, doi:10.1124/pr.110.002832 (2010).

82 Comai, S., Ochoa-Sanchez, R. & Gobbi, G. Sleep-wake characterization of double MT(1)/MT(2) receptor knockout mice and comparison with MT(1) and MT(2) receptor knockout mice. *Behavioural brain research* **243**, 231-238, doi:10.1016/j.bbr.2013.01.008 (2013).

83 Owino, S., Contreras-Alcantara, S., Baba, K. & Tosini, G. Melatonin Signaling Controls the Daily Rhythm in Blood Glucose Levels Independent of Peripheral Clocks. *PloS one* **11**, e0148214, doi:10.1371/journal.pone.0148214 (2016).

84 Alcantara-Contreras, S., Baba, K. & Tosini, G. Removal of melatonin receptor type 1 increases intraocular pressure and retinal ganglion cells death in the mouse. *Neuroscience letters* **494**, 61-64, doi:10.1016/j.neulet.2011.02.056 (2011).

85 Gianesini, C., Hiragaki, S., Laurent, V., Hicks, D. & Tosini, G. Cone Viability Is Affected by Disruption of Melatonin Receptors Signaling. *Investigative ophthalmology & visual science* **57**, 94-104, doi:10.1167/iovs.15-18235 (2016).

86 Weil, Z. M., Hotchkiss, A. K., Gatien, M. L., Pieke-Dahl, S. & Nelson, R. J. Melatonin receptor (MT1) knockout mice display depression-like behaviors and deficits in sensorimotor gating. *Brain research bulletin* **68**, 425-429, doi:10.1016/j.brainresbull.2005.09.016 (2006).

87 Kilic, U. *et al.* Evidence that membrane-bound G protein-coupled melatonin receptors MT1 and MT2 are not involved in the neuroprotective effects of melatonin in focal cerebral ischemia. *Journal of pineal research* **52**, 228-235, doi:10.1111/j.1600-079X.2011.00932.x (2012).

88 Kwon, C. H. *et al.* Neuron-specific enolase-cre mouse line with cre activity in specific neuronal populations. *Genesis* **44**, 130-135, doi:10.1002/gene.20197 (2006).

89 Kugler, S., Kilic, E. & Bahr, M. Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. *Gene therapy* **10**, 337-347, doi:10.1038/sj.gt.3301905 (2003).

90 Wang, X. *et al.* Inhibitors of cytochrome c release with therapeutic potential for Huntington's disease. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 9473-9485, doi:10.1523/JNEUROSCI.1867-08.2008 (2008).

91 Zhu, S. *et al.* Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* **417**, 74-78, doi:10.1038/417074a (2002).

92 Wang, X. *et al.* Methazolamide and melatonin inhibit mitochondrial cytochrome C release and are neuroprotective in experimental models of ischemic injury. *Stroke; a journal of cerebral circulation* **40**, 1877-1885, doi:10.1161/STROKEAHA.108.540765 (2009).

93 Yano, H. *et al.* Inhibition of mitochondrial protein import by mutant huntingtin. *Nature neuroscience* **17**, 822-831, doi:10.1038/nn.3721 (2014).

94 Hersch, S. M. & Ferrante, R. J. Translating therapies for Huntington's disease from genetic animal models to clinical trials. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* **1**, 298-306, doi:10.1602/neurorx.1.3.298 (2004).

95 Ross, C. A. & Tabrizi, S. J. Huntington's disease: from molecular pathogenesis to clinical treatment. *Lancet neurology* **10**, 83-98, doi:10.1016/S1474-4422(10)70245-3 (2011).

96 Meiser, B. & Dunn, S. Psychological impact of genetic testing for Huntington's disease: an update of the literature. *Journal of neurology, neurosurgery, and psychiatry* **69**, 574-578 (2000).

97 Martin, M. *et al.* Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. *Journal of pineal research* **28**, 242-248 (2000).

98 Acuna-Castroviejo, D., Coto-Montes, A., Gaia Monti, M., Ortiz, G. G. & Reiter, R. J. Melatonin is protective against MPTP-induced striatal and hippocampal lesions. *Life sciences* **60**, PL23-29 (1997).

99 Kim, Y. S. *et al.* Melatonin protects 6-OHDA-induced neuronal death of nigrostriatal dopaminergic system. *Neuroreport* **9**, 2387-2390 (1998).

100 Zhang, Y. *et al.* Melatonin inhibits the caspase-1/cytochrome c/caspase-3 cell death pathway, inhibits MT1 receptor loss and delays disease progression in a mouse model of amyotrophic lateral sclerosis. *Neurobiology of disease* **55**, 26-35, doi:10.1016/j.nbd.2013.03.008 (2013).

101 Klein, D. C. Serotonin N-acetyltransferase. A personal historical perspective. *Advances in experimental medicine and biology* **460**, 5-16 (1999).

102 Ackermann, K. & Stehle, J. H. Melatonin synthesis in the human pineal gland: advantages, implications, and difficulties. *Chronobiology international* **23**, 369-379, doi:10.1080/07420520500464379 (2006).

103 Kristian, T. Isolation of mitochondria from the CNS. *Current protocols in neuroscience / editorial board, Jacqueline N. Crawley ... [et al.]* **Chapter 7**, Unit 7 22, doi:10.1002/0471142301.ns0722s52 (2010).

104 Khattar, N. K. *et al.* Isolation of functionally active and highly purified neuronal mitochondria from human cortex. *Journal of neuroscience methods* **263**, 1-6, doi:10.1016/j.jneumeth.2016.01.017 (2016).

105 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408, doi:10.1006/meth.2001.1262 (2001).

106 Carter, R. J. *et al.* Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **19**, 3248-3257 (1999).

107 Menalled, L. B., Lutz, C., Ramboz, S., Brunner, D., Lager, B., Noble, S., Park, L., Howland, D. *A Field Guide to Working with Mouse Models of Huntington's Disease*. (The Jackson Laboratory, 2014).

108 Browne, S. E., Ferrante, R. J. & Beal, M. F. Oxidative stress in Huntington's disease. *Brain pathology* **9**, 147-163 (1999).

109 Crook, Z. R. & Housman, D. Huntington's disease: can mice lead the way to treatment? *Neuron* **69**, 423-435, doi:10.1016/j.neuron.2010.12.035 (2011).

110 Nance, M. A. & Myers, R. H. Juvenile onset Huntington's disease--clinical and research perspectives. *Mental retardation and developmental disabilities research reviews* **7**, 153-157, doi:10.1002/mrdd.1022 (2001).

111 Rattray, I. *et al.* Correlations of behavioral deficits with brain pathology assessed through longitudinal MRI and histopathology in the R6/2 mouse model of HD. *PloS one* **8**, e60012, doi:10.1371/journal.pone.0060012 (2013).

112 Smith, K. M. *et al.* Dose ranging and efficacy study of high-dose coenzyme Q10 formulations in Huntington's disease mice. *Biochimica et biophysica acta* **1762**, 616-626, doi:10.1016/j.bbadis.2006.03.004 (2006).

113 Rigamonti, D. *et al.* Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. *The Journal of biological chemistry* **276**, 14545-14548, doi:10.1074/jbc.C100044200 (2001).

114 Sarang, S. S. *et al.* Discovery of molecular mechanisms of neuroprotection using cell-based bioassays and oligonucleotide arrays. *Physiological genomics* **11**, 45-52, doi:10.1152/physiolgenomics.00064.2002 (2002).

115 Hengartner, M. O. The biochemistry of apoptosis. *Nature* **407**, 770-776, doi:10.1038/35037710 (2000).

116 Gil-Mohapel, J., Brocardo, P. S. & Christie, B. R. The role of oxidative stress in Huntington's disease: are antioxidants good therapeutic candidates? *Current drug targets* **15**, 454-468 (2014).