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The Mechanism of Neuroprotection Mediated By Nicotinamide Mononucleotide Adenylyl Transferase (NMNAT)

Yousuf O. Ali

University of Miami, yoali007@gmail.com

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THE MECHANISM OF NEUROPROTECTION MEDIATED BY NICOTINAMIDE MONONUCLEOTIDE ADENYLYL TRANSFERASE (NMNAT)

By

Yousuf O. Ali

A DISSERTATION

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THE MECHANISM OF NEUROPROTECTION MEDIATED BY NICOTINAMIDE MONONUCLEOTIDE ADENYLYL TRANSFERASE (NMNAT)

Yousuf O. Ali

Approved:

_________________________  ___________________________
Abigail Hackam, Ph.D.  Terri A. Scandura, Ph.D.
Associate Professor of Medicine  Dean of the Graduate School

_________________________  ___________________________
Vladlen Slepak, Ph.D.  Pantelis Tsoulfas, M.D.
Professor and Graduate Program Director  Associate Professor, Department of Neurological Surgery
Molecular and Cellular Pharmacology

_________________________  ___________________________
Michael Kim, Ph.D.  Ming Guo, M.D., Ph.D.
Assistant Professor  Associate Professor, Department of Neurology and Molecular and Medical Pharmacology
Molecular and Cellular Pharmacology

_________________________
Grace Zhai, Ph.D.
Assistant Professor
Molecular and Cellular Pharmacology
Neurons need to be maintained to persist throughout adulthood for proper brain function. However neuronal activity, injury and aging exert physical stress on the nervous system, which compromise nervous system function. Healthy neurons are able to maintain their integrity throughout the lifespan of the animal, suggesting the existence of a maintenance mechanism that allows neurons to sustain or even repair damage. A forward genetic screening in *Drosophila* identified mutations in a gene called *nmnat* that cause a rapid and severe neurodegeneration immediately post neuronal differentiation and development. NMNAT protein was required to maintain neuronal integrity in an activity-dependent manner. When probing for the exact role of NMNAT in neuronal maintenance, a novel stress responsive chaperone function was identified, in addition to its essential housekeeping NAD synthase role. In this work, the mechanism of NMNAT-mediated neuroprotection is investigated. First, the transcriptional regulation of Drosophila NMNAT during acute stress is analyzed. Here, both stress transcription factors heat shock factor (HSF) and hypoxia inducible factor alpha (HIF1-α) have been shown to upregulate NMNAT during stress through a heat shock element in the *nmnat* promoter. In addition, the role of NMNAT for stress tolerance in Drosophila is revealed. Second, to elucidate the
neuroprotective capacity of NMNAT in neurodegenerative disease, mouse models of tauopathy have been used. In the P301L Tau-transgenic mouse model, the levels of endogenous NMNAT2 have been studied at various ages to link a reduction in NMNAT2 as a precursor for neurodegeneration. The underlying mechanism of NMNAT2 downregulation is further studied in this model. Third, using *Drosophila* model of Tauopathy, the protective capacity of both wild type and enzyme-inactive NMNAT in ameliorating the pathological and behavioral impairments from Tau-induced neurodegeneration were studied extensively. The possible protective mechanism of NMNAT is uncovered by identifying novel interactions of NMNAT with hyperphosphorylated and ubiquitinated Tau in regulating the levels of toxic Tau species. Finally, this study also identified endogenous proteins that NMNAT interacts with to provide insight into a neuroprotective chaperone role of NMNAT. Together, these studies improve our understanding of the mechanisms of neuronal maintenance, by providing a comprehensive investigation of the stress-responsive regulation of NMNAT in both Drosophila and mammalian models, and its role as a chaperone both in protein foldopathies and in healthy neurons.
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Chapter 1. Introduction

Neurons need to be maintained to persist throughout adulthood for proper brain function. However, neuronal activity, injury and aging exert physical stress on the nervous system, which compromise nervous system function. Healthy neurons are able to maintain their integrity throughout the lifespan, suggesting the existence of a maintenance mechanism that allows neurons to sustain or even repair damage. In fact, in rodents, long-term confocal microscopy imaging have shown a large fraction of dendritic spines and complete dendritic arbors to be stable for extended time periods of several months, and possibly years (Holtmaat et al., 2005; Lin and Koleske, 2010; Majewska and Sur, 2006; Trachtenberg et al., 2002). These observations argue that individual synapses may last for the vast majority of an organism's lifetime, possibly decades in the case of humans.

Synapse and dendritic spine loss and dendritic atrophy are major hallmarks of the aging human brain and such reductions in synapse number and dendritic arbor size are associated with the majority of psychiatric illnesses such as schizophrenia and major neurodegenerative diseases such as Alzheimer's disease (reviewed in, Lin and Koleske, 2010). Despite the importance of synapse stability for human brain health, a major unmet deficiency in neuroscience is the lack of understanding on how neurons maintain long-term stability in face of continuous activity- and environment-induced stress. Not much is known about essential molecules that provide such maintenance function, whose deficiency in a neuron would be expected not to affect its development but
cause degeneration upon demand for maintenance that occurs with normal neuronal activity.

Recent work in *Drosophila* identified *alicorn*, the *Drosophila* homolog of AMP-activated protein kinase (AMPK) to regulate neuronal maintenance in response to metabolic stress (Spasic et al., 2008). Loss of Alicorn in photoreceptors causes severe early-onset progressive non-apoptotic neurodegeneration in the retina, the optic lobe, and the antenna, as well as neurophysiological and behavioral defects (Spasic et al., 2008). The observed degeneration occurs immediately after the neurons have completely developed and was enhanced with increased light exposure. Conversely, blocking activity by maintaining the flies in the dark blocked the degeneration significantly. In fact, Alicorn was shown to be required to maintain viability of differentiated neurons and preserve their integrity under conditions of increased activity and excitotoxicity (Spasic et al., 2008).

A similar maintenance function was identified in an essential housekeeping enzyme, nicotinamide mononucleotide adenylyltransferase (NMNAT), where complete loss-of-function of the protein caused severe neurodegeneration post development (Zhai et al., 2006; Zhai et al., 2008b). Adjusting the level of activity in the mutant neurons controlled the rate of neurodegeneration (Zhai et al., 2006). The common feature between Alicorn and NMNAT is that both these proteins are required for neuronal maintenance but not
for development. Both of these proteins are required to enable neurons to face the demands of increased activity, and in their absence, neurons are handicapped to survive the demands posed from regular activity. Hence, the key feature of such a maintenance factor is that its demand is enhanced by activity and reduced by blocking activity. Understanding the commonalities between these factors will help identify the components of neuronal maintenance machinery.

1.1 Neurodegenerative Diseases: A Plight for Neuronal Maintenance?

Neurodegenerative conditions are among the most intractable of diseases affecting the aging population. Decades of both clinical and basic science research have discovered and characterized dozens of neurodegenerative disorders, triggered by a variety of genetic and environmental factors. Compared to other cell types, neurons are especially susceptible to degeneration because of their long lifetime (the entire lifespan of the organism for most neurons), and, when damaged or lost, they are not readily replenished through cell division, with the exception of neurons in special neurogenic zones in vertebrates (Merkle and Alvarez-Buylla, 2006). The long life of neurons demands a high level of neuronal maintenance and protection. Reduced synaptic connectivity and dendritic atrophy are major hallmarks of many psychiatric and neurodegenerative diseases (reviewed in, (Lin and Koleske, 2010)). Although there might be a developmental component to defects in initial circuit formation that might lead to such diseases,
most of the impairments manifest in late adulthood. There is an emerging theme that the pathophysiological changes that cause these diseases target synapse and dendrite maintenance (Lin and Koleske, 2010). Exploiting such maintenance pathways that can prolong neuronal survival can facilitate in increasing the therapeutic window for treatment to alleviate neurodegeneration.

Although the knowledge on neuronal maintenance is quite limited and premature, identifying so far only two such molecules, NMNAT and AMPK (alicorn in Drosophila) that affect proper neuronal function and maintenance with regular activity, lack of either of these two maintenance factors have been shown to cause severe activity-dependent degeneration (Spasic et al., 2008; Zhai et al., 2006; Zhai et al., 2008b). These studies provide evidence that deficiencies in neuronal maintenance can directly cause neurodegeneration. In fact, activated AMPK phosphorylated at Thr-172 (p-AMPK) is abnormally and massively accumulated in paired helical filament tau-positive (PHF) neurons in Alzheimer’s disease brains, and in all the major primary tauopathies, including progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), argyrophilic grain disease (AGD), Pick’s disease (PiD), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), tangle-predominant dementia (Vingtdeux et al., 2011). Co-localization was observed in almost 90% of neurons immunoreactive for PHF1 (phospho-Ser-396/404 tau) and CP13 (phospho-Ser-202 tau) (Vingtdeux et al., 2011). AMPK might be critical for the neurodegenerative process of these diseases, although it is unclear whether
AMPK activation is detrimental or neuroprotective in tauopathies. With respect to NMNAT, reducing mammalian NMNAT2 levels in cultured mouse superior cervical ganglia neurons induced Wallerian-like degeneration, suggesting that NMNAT2 is required to maintain axonal health in the peripheral nervous system (Gilley and Coleman, 2010). Interestingly, several gene-array studies found that NMNAT2 levels were reduced in brain specimens from patients with Alzheimer’s disease, Parkinson’s disease and Huntington’s disease (https://www.nextbio.com). Although it is not known whether the consistent decrease in nmnat2 levels is a cause or a result of neurodegeneration in neurodegenerative diseases, over-expression of NMNAT, on the other hand, provides neuroprotection against several degenerative conditions in Drosophila (Zhai et al., 2006; Zhai et al., 2008a).

1.2 Tauopathy as a Specific Example of Neurodegeneration

This chapter gives a detailed overview of tauopathy since part of this thesis focuses on studying the neuroprotective effect of NMNAT in both mouse and Drosophila models of tauopathy. Since the identification of tau as the main component of neurofibrillary tangles in Alzheimer's disease and related tauopathies, and the discovery that mutations in the tau gene cause frontotemporal dementia, much effort has been directed towards understanding how the aggregation of tau into fibrillar inclusions cause neurodegeneration (Hutton, 2000; Hutton et al., 1998; Hutton et al., 2001; Komori, 1999).
Tauopathies characterized by neurofibrillary tangles of phosphorylated tau proteins are a group of neurodegenerative diseases that include frontotemporal dementia and both sporadic and familial Alzheimer’s disease. Hyperphosphorylated microtubule-associated protein tau is the major component of the intracellular neurofibrillary tangles (NFTs) that are tauopathy hallmarks. Tauopathies include several neurodegenerative diseases such as Pick’s disease and fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17; reviewed in (Lee et al., 2001)).

Tau was first isolated in 1975 and shown to bind with tubulin enabling microtubule assembly \textit{in vitro} (Cleveland et al., 1977; Weingarten et al., 1975). As one of the principal components of the cytoskeletal system, microtubules are essential for maintenance of neuronal morphology and formation of axonal and dendritic processes. In addition, microtubules play a very essential role in cellular trafficking by providing tracts for motor proteins, like kinesins and dynein, to ensure transport of cargo to specific parts of the cell. Some of the cargo transported to and from pre- or postsynaptic sites include mitochondria, components of synaptic vesicles and plasma membranes, ion channels, receptors and scaffolding proteins and is essential for synaptic function (Gendron and Petrucelli, 2009). Synapses are especially vulnerable to impairments in microtubule transport as any perturbations in this transport system could lead to problems in neurotransmission and signal propagation and ultimately cause synaptic degeneration (Gendron and Petrucelli, 2009).
Though the gene encoding tau is not genetically linked to Alzheimer’s disease, mutations in tau cause FTDP-17 (Hutton et al., 1998; Spillantini et al., 1998), and missense mutations in tau have also been linked to progressive supranuclear palsy (Delisle et al., 1999), and corticobasal degeneration (Bugiani et al., 1999; Poorkaj et al., 2002), providing evidence that disrupting tau homeostasis is sufficient to cause neurodegeneration. These neurodegeneration-causing mutations have been shown to alter the relative proportion of various tau isoforms (Hutton et al., 1998), impair microtubule binding and assembly (Dayanandan et al., 1999; Hasegawa et al., 1998; Hong et al., 1998), or increase phosphorylation and aggregation of tau into filaments (Goedert et al., 1999; Nacharaju et al., 1999). Hence, the expected cause of tau-mediated neurodegeneration is a combination of toxic gain of function from abnormalities in tau, as well as from the harmful consequences resulting from the loss of normal tau functions.

Various animal models have been developed to study the causes of neurodegeneration induced by overexpression of wild type or mutant tau. Transgenic mice expressing human wild type or mutant tau showed NFT formation and neuronal loss, implying a causal relationship between tau overexpression and neurodegeneration (Andorfer et al., 2005; Andorfer et al., 2003; Gotz et al., 2001; Lewis et al., 2000; Yoshiyama et al., 2007). However, neuronal loss appears to be independent of tangle formation in some transgenic mouse
models (Andorfer et al., 2005; Spires et al., 2006; Wittmann et al., 2001). Although there is evidence for the presence of activated caspase and morphological features of necrosis, autophagy, and abnormal cell cycle events (Andorfer et al., 2005; de Calignon et al.; Gamblin et al., 2003; Gotz et al., 2001; Lewis et al., 2000; Lin et al., 2003; Rissman et al., 2004) the precise mechanism of cell death in tauopathies remains unknown. Much of the tau-mediated toxicity in these models have been linked to hyperphosphorylation of tau that causes it to aggregate into filaments and cause a gain-of-function toxicity. In the disease state, the hyperphosphorylation of tau is about four to eight times higher than in age-matched controls, disrupting its general microtubule binding function and allowing for the formation of neurofibrillary tangles (Alonso et al., 1996; Chatterjee et al., 2009; Khatoon et al., 1992; Ledesma et al., 1996; Lovestone et al., 1999). Investigating the role of phosphorylation states of tau towards neurotoxicity revealed that many phospho-epitopes created by proline directed kinases (SP/TP) sites show relative specificity for disease states (Steinhilb et al., 2007a; Steinhilb et al., 2007b). In fact, blocking phosphorylation by mutating all these SP/TP sites to alanine significantly reduced tau toxicity in vivo (Steinhilb et al., 2007b). Hence, tau toxicity in tauopathies can be directly correlated to its hyperphosphorylated state, where tau loses its normal function such as affecting microtubule stability.

*Drosophila melanogaster* has been used previously as an in vivo model for tauopathy, recapitulating many of the salient features of the disease (Jackson
et al., 2002; Wittmann et al., 2001). Transgenic flies neuronally overexpressing either wild type or mutant human tau, hTau$^{R406W}$, associated with FTDP17, showed adult onset, progressive neurodegeneration with compromised lifespan, and accumulation and hyperphosphorylation of tau (Wittmann et al., 2001). Interestingly, all these disease features are exhibited in Drosophila tauopathy models with minimal formation of insoluble tau aggregates or NFTs (Williams et al., 2000; Wittmann et al., 2001), which are commonly associated with the human disease pathology, thereby suggesting an essential role for the soluble, hyperphosphorylated tau oligomers in the onset and progression of degenerative phenotypes. Furthermore, in these fly models, overexpressing tau in the mushroom body neurons elicited decrements in associative olfactory learning and memory, prior to the initiation of neurodegeneration, indicating that perturbations in behavioral plasticity may be one of the earliest manifestations of tauopathy (Mershin et al., 2004).

1.3 Chaperones Play a Role in Neuronal Maintenance and Homeostasis

Chaperones are the main workforce for cellular maintenance and stress response. Research on neurodegenerative diseases in recent years has uncovered the tremendous neuroprotective properties of chaperones and has since placed chaperones in the center stage of neuroprotection. Hence, stimulating and augmenting the intrinsic chaperone activity in the nervous system has become a main focus in the design of many neuroprotective strategies.
Molecular chaperones are a family of proteins that facilitate and regulate proper protein folding. They do so by binding to and stabilizing proper conformation of client proteins, and, through cycles of regulated binding and release, facilitate their correct fate by preventing inappropriate misfolding (Ellis and Hartl, 1999). Based on their abundance in a cell at any one time, chaperones can be categorized into three groups: 1) constitutively expressed (example, Hsc70, TRiC, peroxiredoxin); 2) constitutively expressed and induced upon stress, (example, Hsp90, NMNAT); and 3) only inducible (upon stress, Hsp70) (Morimoto, 1998, 2008).

Various stress conditions such as heat shock and hypoxia can cause protein misfolding and other cellular damage. Cells respond by transcriptionally activating protective chaperones called the heat shock proteins. This heat shock response is regulated by a family of heat shock transcription factors (HSFs). Under unstressed conditions, HSF1 is maintained in a monomeric state by associating with multi-chaperone complex of Hsp90, Hsp70 and Hsp40 (Abravaya, 1992; Shi, 1998). Upon stress, when there is a demand for chaperones due to protein misfolding, HSF1 dissociates from chaperones (Anckar, 2007) and can trimerize through an extended heptad repeat (HR-A/B) located between the DNA-binding domain and the transcription activation domain (Sorger, 1989). Trimerization is essential for exposing the DNA-binding domain of HSF1 and is followed by nuclear translocation, allowing for binding to heat
shock elements (HSE) in respective candidate gene promoters, leading to upregulation of HSPs (Morimoto, 1998). Binding of HSF1 to respective HSEs releases a preinitiated paused RNA polymerase II complex elongation factors including pTEFb are recruited (Lis, 2000). The presence of stress is a prerequisite for the activation state of DNA-bound HSF1 via various post translational modifications such as phosphorylation (Kline, 1997) and sumoylation (Anckar, 2006). Reduction in stress levels will trigger a negative feedback loop where excess chaperones will sequester monomeric HSF1, thus attenuating HSF1-mediated transcription (Kline, 1997; Knauf, 1996; Sorger, 1988). Therefore, a combination of post translational modifications and protein-protein interactions offers various levels of control on the stress-induced transcriptional activities of HSF1. A schematic diagram of the HSF1 activation is illustrated in Figure 1.3.

Many neurodegenerative diseases are considered “conformational” in nature and are termed “foldopathies” as they are characterized by the accumulation of aberrantly folded proteins (Brown, 2007; Chaudhuri and Paul, 2006; Forman et al., 2004; Muchowski and Wacker, 2005; Selkoe, 2004). Being terminally differentiated, post-mitotic cells, neurons are especially susceptible to the detrimental effects of misfolded proteins as they are unable to reduce the load of toxic intermediates through consecutive rounds of mitosis (Muchowski and Wacker, 2005). Therefore, the capacity of neuronal chaperones to reduce misfolded proteins is essential for maintaining neuronal integrity. Moreover, in
Figure 1.1 Induction of the heat shock response and the expression of neuroprotective chaperones via pharmacological modulation of heat shock

Step 1, under normal conditions, monomeric HSF1 is sequestered in the cytoplasm by constitutively expressed HSPs including Hsp90. Stress conditions activate the transcriptional activity of HSF1 in a stepwise manner. Step 2, Upon physiological stress, HSPs dissociate from HSF1 to interact with misfolded proteins. Step 3, free HSF1 monomers change conformation and trimerize. Step 4, HSF1 trimers translocate to the nucleus. Step 4, upon further activation (e.g. phosphorylation, SUMOylation), HSF1 trimers bind to heat shock elements (HSE) in the promoter/enhancer regions of heat-shock responsive genes. Examples of compounds that promote expression of such genes by acting on the steps shown. Radicocol, geldanamycin and 17AAG inactivate Hsp90 thereby derepressing monomeric HSF1. Sodium salicylate promotes HSF1 trimerization and curcumin promotes trimer translocation. Bimoclomol and arimoclomol enhance the affinity of HSF1 for HSE binding. *(Adapted from Ali YO et al, 2010, Molecules, 15, 6859-6887)*
most neurodegenerative diseases the misfolded protein aggregates strongly co-localize with molecular chaperones (Table 1.3). A recent study comparing the expression levels of Hsc70 in different neuronal subtypes typically vulnerable in neurodegenerative diseases, including spinal motoneurons (vulnerable in amyotrophic lateral sclerosis (ALS)), neurons of the hippocampus/entorhinal cortices (vulnerable in Alzheimer’s disease (AD)), and tyrosine hydroxylase positive neurons of the substantia nigra (vulnerable in Parkinson’s disease (PD)), reported an inverse correlation between relative levels of Hsc70 with the frequency of disease prevalence in the US population (Chen and Brown, 2007).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Associated Genes</th>
<th>Pathology</th>
<th>Aggregate-Associated Chaperones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer’s disease</td>
<td>APP, Presenilin1/2</td>
<td>Extracellular plaques of Aβ40 and Aβ42; Intracellular neurofibrillar tangles (NFTs)</td>
<td>Hsp72, Hsp28, Hsp27, GRP78, Hsp27, Hsp90</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>α-synuclein, Parkin, Pink1, DJ1</td>
<td>Intracellular Lewy Bodies</td>
<td>Hsp70, Hsp40, αβ-crystallin</td>
</tr>
<tr>
<td>Familial ALS</td>
<td>SOD1</td>
<td>Intracellular inclusion bodies</td>
<td>Hsc70</td>
</tr>
<tr>
<td>Spinocerebellar ataxia</td>
<td>Ataxins</td>
<td>Nuclear inclusions</td>
<td>Hsp40, Hsp70</td>
</tr>
</tbody>
</table>

Table 1.1 Association of molecular chaperones with neurodegenerative disorders. Examples of common neurodegenerative diseases with chaperones shown to colocalize with respective protein aggregates either in disease tissue or in experimental models. (Aβ= amyloid beta; APP= amyloid precursor protein; DJ1= Parkinson’s disease (autosomal recessive, early onset); PINK1= phosphatase and tensin induced putative kinase 1; SOD1= superoxide dismutase 1). (Adapted from Ali YO et al, 2010, Molecules, 15, 6859-6887)

Various studies in animal models of neurodegenerative diseases have described neuroprotective effects observed from overexpressing chaperones. Chaperones, such as Hsp70 and Torsin A, a protein with homology to yeast
Hsp104, are colocalized with α-synuclein (αSN) containing Lewy bodies (McLean, 2002) in a mouse model of Parkinson’s disease. Furthermore, Hsp70 was shown to inhibit αSN fibril formation by binding to prefibrillar oligomers and ameliorating toxicity of αSN aggregates (Dedmon, 2005). In Drosophila, overexpression of Hsp70 can dramatically reduced dopaminergic neuronal loss induced by αSN-overexpression, and conversely reducing Hsp70 levels enhanced αSN toxicity (Auluck, 2002). The likely mechanism of Hsp70-mediated protection in Parkinson’s disease model possibly involves the recruitment of misfolded proteins as substrates for parkin E3 ubiquitin ligase and degradation of aberrant αSN (Tsai, 2003). The role of chaperones has also been extensively studied in Alzheimer’s disease models, with respect to tau aggregation and fibrillization. Chaperones, including Hsp27, Hsp70 and CHIP, were shown to colocalize with abnormal tau aggregates, and overexpression of these chaperones reduced tau hyperphosphorylation and increased misfolded tau degradation (Petrucelli et al., 2004; Shimura et al., 2004). PolyQ diseases, such as Huntington’s disease, are among the first protein misfolding diseases in which neuroprotective properties of chaperones have been identified. PolyQ diseases are a family of neurodegenerative diseases caused by proteins containing tandem polyglutamine repeats, which are prone to aggregate. Studies using various model systems have shown that overexpression of Hsp40 and Hsp70 reduced polyQ toxicity and inclusion body formation (Chai, 1999; Jana, 2000). Overexpression of Hsp27 also reduced polyQ toxicity and oxidative stress but did not affect inclusion body formation (Wyttenbach, 2002).
Despite the full repertoire of chaperones in neurons, their importance in neuroprotection only became evident recently. In fact, the high demand of neuronal maintenance and self-protection warrants an indispensable protective role of chaperones in neurons. A failure of such a maintenance system, or toxic stress at levels surpassing the maintenance capacity, would ultimately result in neurodegeneration. Furthermore, compromised activity or function of chaperones would increase the susceptibility of an organism to neurodegeneration. Conversely, harnessing the maintenance and stress response capacity of neurons can increase neurons’ tolerance to internal or external insults. Recent findings that increased neuronal levels of specific chaperones have neuroprotective capacity in several neurodegenerative disease models further emphasize the importance of understanding the regulatory mechanisms of chaperones. As exemplified by the HSF1 pathway, where the neuroprotective properties of several compounds and small molecules have only been revealed after the elucidation of the regulatory mechanisms of HSF1 (Figure 1.3), future studies on dissecting the intricate regulatory network in neuronal chaperones and stress response will not only contribute to the understanding of neuroprotection but also contribute to designing therapies for neurodegenerative diseases.
1.4 NMNAT: A Novel Neuronal Maintenance Factor

Nicotinamide mononucleotide adenylyltransferase has been traditionally known as an essential housekeeping enzyme catalyzing the reversible condensation of ATP with nicotinic acid mononucleotide (NaMN) or nicotinamide mononucleotide (NMN) to produce nicotinic acid adenine dinucleotide (NaAD) or nicotinamide adenine dinucleotide (NAD) (Zhai et al., 2009). NAD is an essential cofactor in many cellular processes such as transcriptional regulation and oxidative reactions. However, recent work has focused a lot of attention on NMNAT for its neuroprotective capacities highlighting novel functions of this protein in conjunction with its NAD synthase activity (MacDonald et al., 2006; Press and Milbrandt, 2008; Sasaki and Milbrandt; Sasaki et al., 2009; Wang et al., 2005; Zhai et al., 2006; Zhai et al., 2008b). The neuroprotective role of NMNAT emerged with the characterizing of the spontaneous mutation in the Wallerian deneneration slow (Wld\textsuperscript{s}) mouse (Lunn et al., 1989). These mice carry a spontaneous dominant mutation that delays Wallerian degeneration in the distal stump of an injured axon. The mutation results in a chimeric gene Ube4b/NMNAT1, containing the entire coding region of mouse NMNAT1, resulting from a random gene triplication (Conforti et al., 2000; Mack et al., 2001). The resulting fusion protein has three characteristic parts: the amino-terminal 70 amino acid fragment of the ubiquitination factor Ube4b, a unique 18 amino acid linker region arising from the 5’ untranslated region (UTR) of NMNAT1 and the full length of NMNAT1 (Conforti et al., 2000; Mack et al., 2001). The dramatic
enhanced neuroprotective effects offered by this mutant protein against various forms of neurodegenerative conditions have drawn a significant amount of attention to uncover the mechanism of protection by this factor. Hence, NMNAT has been studied exclusively to understand if the protection availed from Wld<sup>s</sup> and the various NMNAT isoforms by neurons in varying toxic conditions and injury models is derived from the enzymatic function of this protein or by some novel dual function (MacDonald et al., 2006; Press and Milbrandt, 2008; Sasaki and Milbrandt; Sasaki et al., 2009; Wang et al., 2005; Zhai et al., 2006; Zhai et al., 2008b). In this chapter, both the enzyme function and other novel function of this protein are discussed in detail.

1.4.1 NMNAT is an essential housekeeping enzyme

NMNAT is conserved as an essential housekeeping NAD synthase and despite the lack of sequence identity across species, they share highly conserved structural architecture (Fig. 1.4; (Zhai et al., 2009)). To serve as an NAD-synthase, all NMNATs contain an α/β domain that resemble the common dinucleotide binding domain known as the “Rossman fold”, which provides the structural determinants for substrate recognition and catalysis (Zhai et al., 2009). In fact, structural comparison along with sequence analysis incorporates NMNAT in the nucleotidyltransferase α/β phosphoesterase superfamily with the signature (H/T)XXH sequence required for nucleotide binding (Bork et al., 1995; D'Angelo et al., 2000). There are indeed two of these signature fingerprints in all NMNATs: the (H/T)XXH motif in the N-terminal region and the SXXXXXR motif in
the C-terminal of the protein (Garavaglia et al., 2004). All the conserved residues in the two motifs are required for ATP recognition, which binds in a pocket located at the $\alpha/\beta$ topological switch point between the first and the fourth parallel $\beta$ strand of the central $\beta$ sheet (Zhai et al., 2009). From studies conducted on the prototypical NMNAT from archaea (MjNMNAT) (D'Angelo et al., 2000), the first histidine in the N-terminal motif interacts with the ATP $\beta$-phosphate whereas the second conserved histidine binds $\alpha$-phosphate of ATP and is essential for cata-

Figure 1.2 Ribbon representation of NMNAT from different sources. The structural element, either a long loop or a loop and a small $\beta$ strand that connects the $\alpha/\beta$ domain to the C-terminal domain is depicted in orange. Such a structural element is subjected to conformational changes that accompany catalysis and also provides a key contribution to the oligomeric assembly stabilization in all the NMNATs featured with a quaternary structure, either dimers, tetramers or hexamers. (Adapted from Zhai RG et al, 2009, Cellular and Molecular Life Sciences with permission from Springer Science+Business Media)
-lysis. The C-terminal motif contains a conserved serine residue that is required to form a hydrogen bond with the ATP β phosphate; while the conserved arginine residue is absolutely important for providing salt bridges to connect to ATP γ phosphate through its guanidinium moiety (Zhai et al., 2009). Magnesium is also essential for catalysis since structural analysis has shown close interactions of ATP with it, as the catalytic role of the cation was proposed to be the polarizer favoring the nucleophilic attack on ATP (D'Angelo et al., 2000).

Despite the conservation noted at the level of binding ATP, NMNATs vary in their substrate preference across species: being able to bind either nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN) (Lau et al., 2009; Magni et al., 2004). In fact, depending on the species NMNAT shows preference for either one or both forms of the pyridine mononucleotide. For example, MjNMNAT preferentially binds only NMN while bacterial NMNAT binds only NaMN. At the same time, all human NMNAT isoforms 1, 2 and 3 can bind both substrates with similar affinity (Berger et al., 2005; Sorci et al., 2007). However, despite resolution of crystal structures of different NMNATs in complex with NMN, NaMN, NAD or NaAD, there is no explanation for structural determinants of substrate preference, since the stabilization of the carboxylic, ribose and phosphate moieties is based on peculiar interactions that vary widely between enzymes to make any general predictions (Han et al., 2006; Lu et al., 2008; Olland et al., 2002; Saridakis et al., 2001; Saridakis and Pai, 2003; Sershon et al., 2009; Yoon et al., 2005; Zhang et al., 2002; Zhang et al., 2003). A
strictly conserved Trp residue in all NMNATs is essential for recognition of the pyridine ring through an aromatic stacking interaction (Zhai et al., 2009). Not much is known about the residues responsible for ribose and phosphate recognition, even though a positively charged residue, probably a lysine or an arginine, which appear to be conserved possibly play an important role in recognizing the pyridine mononucleotide phosphate. One plausible explanation for substrate preference comes from studying the archaeal and bacterial NMNAT, where the archaeal NMNAT possibly binds NMN by creating a poorly polar environment less favorable for the negative charge bearing NaMN. Whereas, the bacterial NMNAT prefers NaMN due to the presence of a peculiar anion binding pocket. In summary, the recognition by different NMNATs of either NMN or NaMN is dependent on a series of subtle factors instead of well-defined structural elements (Zhai et al., 2009).

The Mg$^{2+}$ dependent catalysis reaction involves the nucleophilic attack of the pyridine mononucleotide 5'-phosphate on the ATP $\alpha$-phosphate yielding NaAD/NAD and inorganic pyro-phosphate (Lowe et al., 1983). Of particular interest, is the remarkable capacity of the enzyme to catalyze both the forward and the reverse reactions with identical efficacy via a sequential bi-bi mechanism (Berger et al., 2005). In depth analysis of the enzyme kinetics for human NMNAT1 and 2 revealed that ATP binds first followed by NMN, while in product release, pyro-phosphate was first released following NAD (Sershon et al., 2009; Sorci et al., 2007). However, in case of human NMNAT3, NMN was shown to
bind first followed by ATP, while the product release sequence was similar to that of human NMNAT1 and 2 (Werner et al., 2002; Zhang et al., 2003). Such an ordered bi-bi mechanism would allow speculations for specific structural changes in NMNAT upon first substrate binding that can then accommodate the binding of the second substrate.

1.4.2 NMNAT functions as a neuronal maintenance factor independent of its enzyme activity

In the previous section, I have discussed the requirement of NMNAT as an essential housekeeping enzyme, exploring the structural and enzymological bases of its NAD synthesis function. Even though the NAD synthase function of NMNAT is essential for cell function and survival, additional functions of NMNAT have been identified recently that can explain its neuroprotective function in the CNS (Berger et al., 2007; Zhai et al., 2006; Zhai et al., 2008b).

Studies in the Wld<sup>s</sup> mice have already suggested that the chimeric protein Ube4b/NMNAT offers neuroprotection in a variety of neurodegenerative conditions such as injury induced axonal degeneration in peripheral neurons, environmental stress induced degeneration including stress from Taxol and oxidative stress/ischemia, and in genetic mutation-induced neurodegeneration in mice (Charcot-Marie-Tooth disease) and Drosophila (SCA1) (Ali et al.; Araki et al., 2004; Mack et al., 2001; Sasaki et al., 2009; Zhai et al., 2006; Zhai et al.,
2008b). Studies in DRG explants have shown both NMNAT1 and 3 to protect against axon degeneration from injury (Araki et al., 2004; Conforti et al., 2007; Conforti et al., 2011; Sasaki and Milbrandt; Sasaki et al., 2009). Studies in Drosophila suggested that NMNAT also protects from degeneration resulting from injury, excessive light stimulation (increased neuronal activity), environmental stress as well as from genetic mutations associated with overexpression of Drosophila Ataxin or human Ataxin 1 with an expanded polyglutamine tract (Ali et al.; Zhai et al., 2006; Zhai et al., 2008b). In fact, a recent study has shown that knockdown of NMNAT2 in DRG explants results in neurodegeneration without any external stimuli (Gilley and Coleman). All these studies implicate NMNAT in a neuronal maintenance role absolutely essential in protecting neurons from both internal and external insults.

Studies in Drosophila showed that enzyme-inactive NMNAT displayed neuroprotective capabilities indicting the possibility of an enzyme-independent pathway that might be at work in parallel to its enzyme function (Zhai et al., 2006; Zhai et al., 2008b). Exploring the function of enzyme-inactive NMNAT led to the discovery of a novel chaperone function of the protein. (Zhai et al., 2008b) In measuring the in vivo chaperone activity of NMNAT, an assay in which refolding of heat-denatured luciferase is monitored as a measure for chaperone activity, showed that both wild type and enzyme-dead NMNAT could protect luciferase from unfolding during heat shock and enhance refolding after heat shock (Zhai et al., 2008b). In fact, the chaperone activities of all forms of NMNAT were very
similar to that of mammalian Hsp70 or *Drosophila* Hsp83, the homologue of mammalian Hsp90 (Zhai et al., 2008b). These data indicated that NMNAT protects proteins from unfolding and promotes refolding, either by acting as a chaperone or by regulating the activity of other chaperones. To differentiate between these two possibilities, an *in vitro* biochemical assay that measures the chaperone's ability to reduce thermally or chemically induced aggregation of a model protein substrate such as citrate synthase or insulin, showed that Incubation of the substrate with increasing amounts of NMNAT results in a concomitant decrease in citrate synthase aggregation from thermal denaturation (Zhai et al., 2008b). The dynamics of the concentration-dependent decrease in substrate denaturation was similar to that of Hsp70, a positive chaperone control. At the same time enzymatically inactive NMNAT was able to suppress protein aggregation effectively further suggesting that the chaperone activity of NMNAT is independent of its NAD synthesis activity (Zhai et al., 2008b).

The chaperone function of NMNAT is consistent with its observed neuroprotective capacity, since other chaperones such as Hsp70, Hsp40 and Hsp16.2 have been shown to confer protection in various models of Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease and other poly-glutamine expansion disorders (Auluck and Bonini, 2002; Auluck et al., 2002; Chan and Bonini, 2000; Chan et al., 2000; Cummings et al., 1998; Cummings et al., 2001; Fonte et al., 2008; Gifondorwa et al., 2007; Magrane et al., 2004). The main mechanism of behind protection in all these
diseases is protein misfolding as all these fall under the category of proteinopathies, where the disease is linked to the misfolding of a major protein that results in disruption of normal neuronal maintenance from either toxic effects of the misfolded mutant protein or from a lack of normal candidate protein function.

Concurrent with the molecular chaperone function of NMNAT, extensive structural analysis reveals that NMNAT might share structural elements with other known chaperones that can support its dual chaperone function (Zhai et al., 2009; Zhai et al., 2008b). A DALI search using the structural coordinates of NMNAT1 (Protein Data Bank identity code 1KKU) and NMNAT3 (identity code 1NUR) against the entire Protein Data Bank revealed that both NMNAT1 and NMNAT3 share similar structural components with the bacterial chaperone universal stress protein A (UspA; identity code 1JMV), with a Z score of 5.1, and Hsp100 (identity code 1JBK), with a Z score of 2.8 (Zhai et al., 2009; Zhai et al., 2008b). A Z score of greater than 2 indicates significant structural similarity. When these structures are superimposed, the most significant similarities are observed between NMNAT1 and UspA at the three helices where the folds are overlapping (Fig 1.5). These structural analyses indicate that NMNAT has structural features that can explain its chaperone function.
Further support of this novel chaperone function of NMNAT comes from analysis of its transcriptional regulation upon acute stress paradigms. Infact, *Drosophila* NMNAT is shown to be upregulated at both the transcript and the protein levels upon heat, hypoxia and oxidative stress (Ali et al., 2011). Molecular chaperones, such as the heat shock proteins, are known to be upregulated by these diverse stress conditions. As cells are faced with abnormal conditions, such as heat, oxidative stress, hypoxia, or accumulation of aberrant proteins, cells implement a stress response program to protect themselves and ensure survival. Stress conditions can cause protein unfolding, misfolding, or aggregation, and the
consequent inadequate response to stress can lead to developmental defects, shortened lifespan, and neurodegenerative conditions (Powers et al., 2009). The increased synthesis of molecular chaperone heat shock proteins (HSPs) is central to the stress response because they function to prevent protein misfolding and aggregation to maintain protein homeostasis (Morimoto, 1998; Powers et al., 2009). It is thought that elevated expression of HSPs is sufficient to protect cells from a wide range of cytotoxic conditions (Lindquist and Craig, 1988; Morimoto and Santoro, 1998; Morimoto et al., 1992).

Similar to HSPs, upon acute stress, Drosophila NMNAT is also transcriptionally regulated by the binding of heat shock factor to a proximal element in its promoter region. Heat shock factors (HSFs) are the master stress transcription factors of heat shock response, with one HSF in invertebrates and multiple HSFs in plants and vertebrates (Akerfelt et al., 2007; Nover and Miernyk, 2001; Wu, 1995). Through their roles in mediating transcriptional activation of HSP genes, HSFs function in maintaining protein homeostasis and integrating cellular response to stress and development (Akerfelt et al., 2010). Upregulation of HSPs by HSF1 is triggered by a variety of acute and chronic stress conditions and disease states (Ali et al., 2010).

However, the chaperone function of NMNAT may not explain all of the neuroprotective effects observed with NMNAT overexpression. Studies in DRG explants have shown exogenously provided NAD may be neuroprotective post
injury. However, there were discrepancies among different studies, whereby one study showed that applying NAD is neuroprotective, although the effect is not specific and can be availed by providing pyruvate or EGTA (Araki et al., 2004; Sasaki et al., 2006; Sasaki and Milbrandt). Another study indicated that providing exogenous NAD has no protective effect on axon degeneration in DRGs post injury (Conforti et al., 2007). Even in the Wld<sup>s</sup> mice, where there is more NMNAT1 protein, the NAD levels are surprisingly not elevated (Mack et al., 2001). Moreover, manipulating other enzymes upstream in the NAD synthesis pathway have failed to offer any neuroprotection (Avery et al., 2009; Sasaki et al., 2006). All these studies suggest that NAD synthase activity of NMNAT is only partially responsible for NMNAT’s neuroprotective role. In fact, it is possible that providing exogenous NAD is simply neuroprotective because it frees up NMNAT to perform its other enzyme-independent functions.

Apart from the NAD synthase and chaperone function of NMNAT, recent work has shown that NMNAT1 can significantly affect nuclear polyADP-ribosylation by directly interacting with poly(ADP-ribose) polymerase 1 (PARP-1) (Berger et al., 2007). PARP-1 is an abundant nuclear enzyme that binds to DNA single-strand breaks that triggers its catalytic activity, the synthesis of ADP-ribose polymers, thereby initiating events such as the recruitment of DNA repair proteins (D'Amours et al., 1999; Dantzer et al., 2000; Virag and Szabo, 2002; Vohra et al., 2010). The NMNAT1:PARP-1 interaction initiates PARP-1 automodification, thereby increasing the extent of poly(ADP-ribose)ylation. Moreover, specific
phosphorylation of NMNAT-1 by protein kinase C (PKC) prevents this interaction hampering its regulation of PARP-1 activity (Berger et al., 2007). Poly(ADP-ribosyl)ation is known to be an immediate early event that follows genotoxic assault (Berger et al., 2007). The interaction of NMNAT1 with PARP-1 might be beneficial in explaining some of the neuroprotection provided by NMNAT1 against environmental stress. In fact, identifying such endogenous binding partners of NMNAT can explain the neuroprotective effects of this protein.

In this study, some of the mechanisms of neuroprotection mediated by NMNAT were investigated, including a stress-dependent regulation of NMNAT and its role in neurodegenerative diseases using both mouse and Drosophila models, and identification of endogenous client proteins of this abundantly present chaperone. Together, these studies improve the current understanding of the mechanisms of neuronal maintenance, by providing a comprehensive investigation of the stress-responsive regulation of NMNAT in both Drosophila and mammalian models, and its role as a chaperone both in protein foldopathies and in healthy neurons.
2.1 – Animal Models

2.1.1 – Fly Stocks

The following *Drosophila* lines were obtained from the Bloomington Stock Center: *hsf4/CyO; cn hsf4; P(hsf^{t8})1/TM3, Sb1Ser^{1}; Tubulin-GAL4; GMR-GAL4; bbg-GAL4; UAS-HSF-RNAi; Df(3R)Hsp70ADf(3R)Hsp70B/Tm6; elav-GAL4; yw; UAS-CD8GFP*. For our Tauopathy model, human wild type tau or Arg^{406}→Trp (R406W) mutant tau containing no N-terminal inserts and four microtubule binding domains (0N,4R) (a gift of Dr. MB Feany), was expressed with a pan-neuronal driver *elav-GAL4*. Fly strains used in experiments that were generated in the lab included *w;; UAS-NMNAT, w;; UAS-NMNAT^{WR}, w;; nmnat/TM6B*. *UAS-HSF-eGFP* line was a gift of Dr. JT Lis (Department of Molecular Biology and Genetics, College of Agricultural and Life Sciences, Cornell University). *UAS-Sima* and *Sima/TM3* lines were gifts from Dr. P. Wappner (Instituto Leloir and FCEyN, Universidad de Buenos Aires, Argentina).

2.1.2 – Fly Culture Conditions

Flies were maintained on a cornmeal-molasses-yeast media and at room temperature (22°C) with 60-65% humidity. For APSA (learning and memory assay), flies were starved for 6 hours prior to assaying PC0 and PC6.
2.1.3 – Mouse Model of Tauopathy

In the tauopathy study, mice over-expressing the P301L mutation in 4R0N human tau associated with FTDP-17, was used. The generation of rTg(\(\text{tau}_{P301L}\)4510 mice (abbreviated as rTg4510 mice) has been described previously (Ramsden et al., 2005; Santacruz et al., 2005). In brief, rTg4510 mice were generated by F1 crossing of responder and activator transgenic lines. Responder mice carried \(\text{tau}_{P301L}\) cDNA with an upstream tetracycline-operon responsive element (TRE) and were maintained on a FVB/N background. Activator mice contained a trans-activator gene consisting of the tetracycline-off open reading frame placed downstream of the calcium-calmodulin kinase II (CaMKII) promoter and were maintained on a 129Sv6 background. Both transgenic lines were gifts from Michael Hutton (Mayo Foundation) and Karen Ashe (University of Minnesota Medical School). The resulting F1 progenies from the cross between activator and responder mice were on a mixed 129Sv6 x FVB/N genetic background. Littermates lacking both the activator and the responder transgenes were used as controls. Mouse genotypes were determined from tail biopsies using real time PCR with specific probes designed for each gene by a commercial service (Transnetyx, Cordova, TN). Animal housing and use were in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the institutional animal care committee at Baylor College of Medicine.
2.2 – Development of Acute Stress Models of Drosophila

2.2.1 – Heat Stress Model
In this model, wild type (yw) flies were heat shocked at 37°C for one hour, acclimatized to room temperature for an hour and then further heat shocked for an hour. Following shock, fly samples were collected at various time points (0, 6, 12 and 24 hours post heat shock) to examine the induction of different molecules at both the mRNA level (Quantitative Real Time PCR) and the protein level quantitatively (Western Blotting).

2.2.2 – Oxidative Stress Model
Here, wild type flies (genotype: yw) were sexed 24 to 48 hours post-eclosion and fed paraquat. Multiple concentrations of paraquat (0mM, 5mM, 10mM and 20mM) in 1% agar medium were used in this study. About 100 male or female flies were typically used for each drug concentration. Similarly, we used the same model to study NMNAT induction and regulation in various transgenic flies. In order to induce oxidative stress in vitro, we used Drosophila Schneider (S2) cells, grown to confluency and treated with 100uM Deferoxamine (DFO), an iron chelator that has been shown to induce oxidative stress in these cells (Centanin et al., 2008; Romero et al., 2008).
2.2.3 – Hypoxia Model

Wild type flies (genotype: *yw*) were collected 24 to 48 hours post eclosion and treated for 2, 6, 12 or 24 hours in 5% O\textsubscript{2} using a calibration gas mixture (4.94% CO\textsubscript{2} and N\textsubscript{2} balance) (Airgas South Inc, Kennesaw, GA).

2.3 – Quantification of mRNA and protein levels

2.3.1 – RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from fly heads by TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), according to manufacturer’s protocol. Briefly, for each group, 40 female fly heads were homogenized in 1mL TRIzol reagent and mixed with 0.2 volumes of chloroform and incubated at room temperature for 5 minutes. The samples were centrifuged at 13,000 rpm for 15 minutes at 4°C, after which the upper phase was removed to a new RNase-free tube. To this was added 0.7 volumes ethanol (70%) and shaken vigorously. The samples were then loaded onto RNeasy QIAprep filters and the RNA purified and isolated according to manufacturer’s protocol (Qiagen, Valencia, CA). For each extraction, RNA concentration was determined spectrophotometrically at 260 nm, and 2 µg RNA was used for reverse transcription reaction with SuperScript First-Strand Synthesis system (Invitrogen Life Technologies, Carlsbad, CA).
Quantification of *Drosophila nmnat* and *hsp70*, and murine *nmnat2* mRNA was performed using an ABI PRISM 7000 sequence Detection System (PE Applied Biosystems, Weiterstadt) and Taqman Universal PCR Mastermix (Applied Biosystems, New Jersey, USA). A housekeeping gene, *rp49 (Drosophila)* and *gapdh* (mouse) were used as an internal controls to standardize mRNA expression. Amplification mix (25µL) contained 100ng cDNA template, 12.5µl Taqman Universal PCR Master mix, and 1µl gene-specific Taqman probe-primer set. The specific primer-probe set acquired from Applied Biosystems are shown below: *Dm nmnat* (*Dm02144514_g1*), *Dm hsp70* (*Dm01839211_g1*), *Dm rp49* (*Dm02151827_g1*), *Ms nmnat2* (*Mm00615393_m1*), *Ms gapdh* (*Mm99999915_g1*). Samples were amplified by a PCR program of 40 cycles of 10s at 95°C, 15s at 55°C and 1min at 72°C. The C\textsubscript{T} value was defined as the number of cycles required for the fluorescence to exceed the detection threshold and the data analyzed using the $2^{-\Delta\Delta Ct}$ method, to quantitatively assess relative changes in gene expression (Livak and Schmittgen, 2001). Briefly, the raw Ct values were averaged for animals per group for both gene of interest (for example, *nmnat2*) and the respective housekeeping control (for example, *gapdh*). $\Delta$Ct values were calculated by subtracting the average “*housekeeping gene*” Ct value from that of the gene of interest. The relative expression of “*the gene of interest*” (GOI) in treated or transgenic animals compared to controls was obtained by calculating the $\Delta\Delta$Ct value for each group by subtracting the average $\Delta$Ct value of GOI of control animal from average $\Delta$Ct of GOI of treated or
transgenic animal. The relative fold change of treated/transgenic groups to control was expressed as $2^{\Delta\Delta CT}$.

2.3.2 – Protein Extraction and quantitative Western Blotting

Proteins were extracted from fly heads with a homogenizing buffer (20mM HEPES pH 7.5, 100mM KCl, 5% Glycerol, 10mM EDTA, 0.1% Triton, 1mM DTT, 0.5mM PMSF, Protease Inhibitor Cocktail (Sigma)). For protein extraction from mouse brain tissue, a different lysis buffer was used consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM PMSF, 1% Triton-X100, protease inhibitor cocktail (Sigma, St Louis, MO), and phosphatase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Total protein concentration was measured using the Bradford Reagent (Thermo Scientific/Pierce Biotechnology, Rockford, IL). Samples were resolved in SDS-PAGE and transferred to nitrocellulose membrane. After incubation in blocking buffer, membranes were incubated overnight in primary antibody solution at 4°C (for details, see Table 2.3). Western blot analysis was performed with infrared dye-conjugated secondary antibodies, IR700 and IR800 (LI-COR Biosciences); blots were imaged and processed on an Odyssey Infrared Imaging System.

2.4 Immunostaining

2.4.1 Clonal Analysis of Transcription Factors

L3 larval imaginal eye and wing discs were fixed in phosphate-buffered saline (PBS) with 3.7% formaldehyde for 15 min and washed in phosphate-buffered
Table 2.1 Antibodies used For Western Blotting and Immunostaining

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Source</th>
<th>Primary Antibody Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Western Blot</td>
</tr>
<tr>
<td>Acetylated Tubulin</td>
<td>Abcam</td>
<td>1:2000</td>
</tr>
<tr>
<td>Actin</td>
<td>Sigma</td>
<td>1:5000</td>
</tr>
<tr>
<td>Caspase3</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cleaved-Caspase3</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
<tr>
<td>CREB</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>GFAP</td>
<td>DAKO</td>
<td>1:4000</td>
</tr>
<tr>
<td>HSF</td>
<td>Gift: Dr. JT Lis</td>
<td>1:1000</td>
</tr>
<tr>
<td>HSP70</td>
<td>Stressgen</td>
<td>1:1000</td>
</tr>
<tr>
<td>hTAU</td>
<td>DAKO</td>
<td>1:1000</td>
</tr>
<tr>
<td>nc82</td>
<td>DSHB</td>
<td>1:500</td>
</tr>
<tr>
<td>NMNAT (Dm)</td>
<td>Lab</td>
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</tr>
<tr>
<td>NMNAT1</td>
<td>Abcam</td>
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</tr>
<tr>
<td>NMNAT2</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>NMNAT3</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>Neurofilament-M</td>
<td>Encore</td>
<td>1:4000</td>
</tr>
<tr>
<td>pCREB (Ser133)</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>PHF-Tau^Ser202/Thr205_</td>
<td>ThermoScientific</td>
<td>1:1000</td>
</tr>
<tr>
<td>PHF-Tau^Thr231</td>
<td>ThermoScientific</td>
<td>1:1000</td>
</tr>
<tr>
<td>phosphoTau^Ser262</td>
<td>Millipore</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

saline with 0.4% Triton X-100. Primary antibody dilutions used are noted in Table 2.3 and secondary antibodies conjugated to Alexa 555 or Alexa 647 (Jackson ImmunoResearch, West Grove, PA; Molecular Probes, Eugene, OR) were used at 1:250. All antibody incubations were performed at 4°C overnight in the presence of 5% normal goat serum.
2.4.2 hTau-induced Vacuole Volume Analysis

Adult brains were fixed in phosphate buffered saline (PBS) with 4% formaldehyde for 20 min and washed in phosphate buffered saline with 0.4% Triton X-100. Antibody dilutions used: anti-NMNAT 1:800; mAb nc82 1:200 (DSHB, University of Iowa, IA); anti-Tau 1:200 (DAKO); anti-Tau\textsuperscript{pSer262} 1:200 (Millipore, Billerica, MA) (Table 2.1). Secondary antibodies conjugated to Alexa-488, -555 and -647, Cy3, or Cy5 (Jackson ImmunoResearch, West Grove, Pennsylvania, United States; and Molecular Probes) were used at 1:250. All primary antibody incubations were performed at 4 °C overnight or secondary for 2 hours at room temperature, in the presence of 5% normal goat serum.

2.4.3 Microtubule stability analysis

Exponentially growing COS-7 were plated on coverslips and transfected the next day with either plenty-mCherry or plenty-NMNAT or plenty-NMNAT\textsuperscript{H30A} using lipofectamine reagent (Invitrogen, Carlsbad, CA), as described by the manufacturer. After 48 hours post-transfection, cells were treated with 10 μM nocodazole for indicated times before processing cells for immunofluorescence with anti-acetylated tubulin (Abcam, 1:200) (Hergovich et al., 2003). DNA was stained with DAPI. At least 300 cells were analysed for an intact microtubule network in each experiment for each construct and each time point. Only cells with intact nuclei and a clearly detectable signal of mCherry were included in the evaluation. Experiments were repeated as blind assays.
2.4.4 Image Processing and Analysis

Images from fluorescently labeled specimens were taken on an Olympus IX81 confocal microscope and processed using FluoView10-ASW (Olympus) and Adobe Photoshop CS4 (Adobe Systems, San Jose, California, United States). Vacuole volume was quantified by using FluoView10-ASW (Olympus) software, assuming each vacuole to be spherical in form. Numbers of brains analyzed per genotype per age for the tau study are noted at the bottom of the bars in Figure 5.2.

2.5 – Cloning and Site Directed Mutagenesis

2.5.1 – Dm nmnat Promoter Cloning and mutagenesis

To investigate the functional binding of Hsf and Hif1-α to the Dm nmnat promoter, 1.5Kb-upstream of the transcription start site (TSS) was cloned into a minimal promoter luciferase vector (PGL4-) using the following primers: PGL4-NMNAT (Forward): 5'-GCTAGCGAAAAGGGTCACAAGTTCG-3'; PGL4-NMNAT (Reverse) 5'-AAGCTTTCCAACACTAAACAGCTGTGC-3'. Failsafe PCR kit (EpiCenter Technologies) was used with the following amplification conditions: 25 cycles of 50s at 95°C, 50s at 61°C and 1min at 72°C. Moreover, the region containing HSEp was also cloned into the same vector using the same reverse primer and a forward primer: 5'-GCTAGCTGGTGTGTGCAACTGAAGGTGC-3' using the same cycling parameters. The PGL4-NMNAT plasmid was used as a template to mutate either the Hif1-α binding site: ACGT to GCAT (FORWARD: 5'-
GTGCAGGCGGGCATCAAGGTCTCG-3'), or the HSeD: GAA to AAG (Forward: 5'-CATACCGCCCACACGCAGCGTTGAACGTCGAAATC-3'; Reverse: 5'-GATTTCGACGTTCAAACGCTTTGCGTGTTGGGCCTAGTATG-3') or the HSeP: GTA to ATG (Forward: 5'-CTAATGTAACACTAATGTCGTCGGGCAATGTCGTTG-3'; Reverse: 5'-GAACGATTGCCTGACGACATTAGTGTTATCGATTAG-3') were mutated either alone or in various combinations using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, CA), according to the manufacturer’s protocol. The following PCR cycling conditions were used for 18-cycles reaction: 30s at 95°C, 60s at 61°C and 5min at 68°C.

2.5.2 – nmnat2 Promoter Cloning and Mutagenesis

In order to study the functionality of the putative CREs indentified in the nmnat2 promoter region, a 2-Kb and 700-bp fragment preceding the transcriptional start site (TSS) of nmnat2 was cloned into a minimal PGL4 luciferase vector. Mouse genomic DNA was used as an amplification template using a Failsafe PCR Kit (EpiCenter Technologies) with the following amplification conditions: 25 cycles of 50s at 95°C, 50s at 61°C and 1min at 72°C. PCR amplification primers include: 2KB-Pro (Forward) 5'-GCT AGC ATA ATT ATG TTA ATT GCC CAG CTT CT, Pro-0.7Kb (Forward) 5'-CTA GCT AGC ACC CAT AAT TCA ACC CAC ACA AAG GAC CAA C-3', 2Kb/700bp-Pro (Reverse) 5'-CCC AAG CTT GGA CTC ACC GAA CAT CTG AAT GTG CCC TTT A-3'. For mutagenesis of the CREs, the 700bp-Pro-PGL4 vector served as the template using the QuikChange II XL
Site-Directed Mutagenesis Kit (Agilent Technologies, CA), according to the manufacturer's protocol. In both the CREs, the 5’-TGACGC-3’ core sequence was mutated to 5’-TGGGTA-3’. The following PCR cycling conditions were used for 18-cycles reaction: 30s at 95°C, 60s at 61°C and 5min at 68°C. The following primers were used for mutagenesis: mCRE1 (Forward) 5’-GAA CCA AGA CCA GTG GGT AAA AAG AAG CTA GAG AGG-3’, mCRE1 (Reverse) 5’-CCT CTC TAG CTT CTT TTTA CCC ACT GGT CTT GGT TC-3’, mCRE2 (Forward) 5’-GAG GCG GGG AGT GGG TAG GTT TGC GTC TAG AG-3’, mCRE2 (Reverse) 5’-CTC TAG ACG CAA ACC TAC CCA CTC CCC GCC TC-3’.

2.6 – Luciferase Assays

2.6.1 – Testing functional Hsf or Hif1-α Binding to Dm nmnat promoter

Drosophila S2 cells were transfected using Cellfectin Reagent (Invitrogen) with pGL4-Renilla Luciferase and one of the following plasmids: pGL4-CMV-Luciferase, pGL4-nmnat\textsuperscript{Pro}-Luciferase, pGL4-HRE-Luciferase, pGL4-HRE+mHSEs-Luciferase, pGL4-HSEp+d-Luciferase, pGL4-mHSEp+d-Luciferase, pGL4-mHSEp-Luciferase, pGL4-mHSEd-Luciferase or pGL4-vegf\textsuperscript{HRE}-Luciferase (gift of Dr. Keith Webster). Forty eight hours after transfection, cells were either heat shocked for 15 minutes (for HSE) or treated with 100 µM DFO (HRE) and 12 hours later lysed and assayed for luciferase activity using the Dual-Glo Luciferase System (Promega), by means of luminescence measurements using a BMG plate reader.
2.6.2 – Testing functional CREs in Ms nmnat2 promoter

293T cells were double transfected using Lipofectamine Reagent (Invitrogen) with a Renilla luciferase vector, -36prl-Renilla luciferase (a gift from Dr. Michael Kapiloff, Univ. of Miami), and a firefly luciferase vector of either nmnat2 promoter-PGL4 luciferase (2Kb-Pro or 700bp-Pro containing both CREs), mCRE1:CRE2-PGL4 luciferase (CRE1 mutated only), CRE1:mCRE2-PGL4 luciferase (CRE2 mutated only), mCRE1:mCRE2-PGL4 luciferase (both CRE mutated) or -36prl-CRE- luciferase (gift of Dr. Michael Kapiloff). -36prl-CRE- luciferase vector contains three copies of CRE in front of the -36bp prolactin minimal promoter and was used as a positive control. In addition to these vectors, cells were additionally transfected with pcDNA-CREB or pcDNA-CREB$^{S133A}$, a dominant negative pCREB mutant. Forty-eight hours after transfection, cells were treated with either DMSO or 5 µM forskolin as described (Chen et al., 2007) for 12 hours. Cells were then lysed and assayed for luciferase activity using the Dual-Glo Luciferase System (Promega, Madison, WI) by luminescence measurement using BMG Omega® plate reader (Imgen Technologies, Alexandria, VA).

2.7- Chromatin Immunoprecipitation Assay

2.7.1 – HSE and HRE binding studies in Dm nmnat promoter

ChIP assay was performed on chromatin obtained from S2 cells using EZCHIP Kit (Upstate), according to manufacturer’s protocol. For HSE binding ChIP, S2 cells were heat shocked at 37°C for 15 minutes. For HRE binding ChIP, S2 cells were transfected with HA-Sima and HA-Tango (Gift of Dr. Pablo Wappner) and
48 hours later treated with 100µM DFO overnight (Romero et al., 2008). Following crosslinking, the chromatin was sheared using a Branson Sonifier 150, with seven 20 second-bursts at high setting (5) with a one minute hold in ice after shearing. Immunoprecipitation was followed with antibodies for either anti-HA (Millipore), anti-HSF (a gift of Dr. J T Lis), anti-RNA PolII (Upstate), and mouse IgG (Upstate). Reverse crosslinking and elution were performed according to the manufacturer's protocol.

PCR of the ChIP DNA was performed using the Failsafe PCR Kit (EpiCenter Technologies) using the following primers:

- **DmNMNAT-HS Ep** Forward: 5′-CGGCGAAGAAAAACAAAAACAAGC-3′; Reverse: 5′-TTCGACGGTGCCGTGCG-3′;
- **DmNMNAT-HS Ed** Forward: 5′-CTCTAGCCAGGGCGGTCTCT-3′; Reverse: 5′-AACATTGCTTTGCTTG-3′;
- **DmNMNAT-HRE** Forward: 5′-AAAAGGTCACAAGTTCG-3′; Reverse: 5′-AAAAGGTCACAAGTTCG-3′;
- **DmHSF70-HSE** Forward: 5′-TGCCAGAAAGAAAAACTCGAGAAA-3′; Reverse: 5′-GACAGAGTGAGAGCAATAGTACAGAGA-3′;
- **RP49 Promoter** Forward: 5′-CCCTGATAAGGATTGATGCTG-3′; Reverse: 5′-CCCTGATAAGGATTGATGCTG-3′;
- **DmHSF-HRE** Forward: 5′-CTCCCACCATACCCGCTAATC; Reverse: 5′-CTCCCACCATACCCGCTAATC;
- **DmHph-HRE** Forward: 5′-AAAAGCCAAGTGAATGACCAAGG; Reverse: 5′-AAAAGCCAAGTGAATGACCAAGG;
- **DmHph-HRE** Forward: 5′-CCTTCTCATACCTCCTCGCTG; Reverse: 5′-CCTTCTCATACCTCCTCGCTG.
CACTCTCTGCCAAGCCAAACC), Actin5c Promoter Forward: 5′-
TGTGTGTGAGAGACGAAAGCC; Actin5c Promoter Reverse: 5′-
CTGGAATAAACGACTGAAAGTGG).

2.7.2 – CRE binding studies in Mm nmnat2 promoter

For ChIP analysis, both wildtype mouse brain and 293T cells were used to investigate pCREB binding to the nmnat2 promoter. ChIP assay was performed on chromatin obtained from brain lysate or 293T cells using EZCHIP Kit (Millipore, Part no. 17-409), according to manufacturer’s protocol.

For wild type mouse brain samples, the crosslinking and shearing procedure was followed according to previously published methods (Dahl and Collas, 2008a, b). Briefly, whole brains from 1 month old mice were finely minced, washed in 2× volume of 1× PBS, and fixed in 1/10 volume of fresh 11% formaldehyde for 10 min at RT. Formaldehyde was then quenched by adding 1/20 volume of 2.5M glycine and cells were then homogenized by a mechanical homogenizer and passed through 100-mm nylon cell strainer. Cells were pooled and spun at 1,100g for 5 min at 4 °C and resuspended in 50 ml 1× PBS by gentle inversion and spun at 1,100g for 5 min at 4 °C to pellet the cells. The final cell pellet was resuspended in 10 ml of 1× PBS, spun at 1,350g for 5 min at 4 °C. Samples were lysed in three different lysis buffers and sonicated (Microson ultrasonic cell disruptor) to shear DNA to an average length of 500-700 bp. 1%
Triton X-100 was added to the sonicated lysate which was then centrifuged at 20,000g for 10 min at 4 °C to pellet debris. After the crosslinking and shearing, the ChIP assay was performed according to the EZCHIP Kit protocol (Millipore, Part no. 17-409).

For 293T cells, in order to perform ChIP, cells were transfected with the 2KB-nmnat2 promoter-PGL4 vector, since prior work shows lack of NMNAT2 expression in 293T cells. Forty-eight hours after transfection, cells were treated with either DMSO or 5µM forskolin before crosslinking proteins with chromatin using formaldehyde. Following crosslinking, the chromatin was sheared using a Branson Sonifier 150, by seven 20-second bursts at high setting (6) with a one minute incubation on ice after each sonication.

Immunoprecipitation was performed with the following antibodies: anti-pCREB (Millipore, Part no. 17-10131), Rabbit IgG (negative control; Millipore, Part no. 17-409) or anti-RNAPolIII (positive control; Millipore, Part no. 17-409). PCR of the ChIP DNA was performed using the Failsafe PCR Kit (EpiCenter Technologies) using the following primers: *nmnat2* CRE1+CRE2 (Forward) 5’-GCT GTA AGG ATG CCA GGG-3’; *nmnat2* CRE2 (Forward) 5’-GCC AAA GGG AGA GCA ATA-3’; *nmnat2* CRE1+CRE2 (Reverse) 5’-CAG CAG GAT AAC GTG GGT-3’. For a positive control, amplification primers included in the ChIP Ab+™
Phospho-CREB (Ser133) antibody kit (Millipore, Part no. 17-10131) were used to amplify cFos CRE (Millipore, Part No. CS203203).

2.8 – Behavioral Assays

2.8.1 – Aversive Phototaxis Suppression Assay (APSA)

The APS assay was performed as described previously (Ali, 2011; Le Bourg and Buecher, 2002). In this assay, each female fly per genotype was individually conditioned to associate the bitter taste of quinine with light and were then tested in a T-maze (See Figure 2.8.1) and allowed to choose between a dark and a lighted vial. Each positively phototactic fly is trained in ten trials to move into the lighted vial, which contains the quinine-soaked filter paper. Repetitive training reinforced them to make the aversive association. Immediately after training, the fly was tested in a series of five trials to see if they learned to make this association (PC0; learning function). These flies were again tested 6 hours later to see if they remember to make this negative association of bitter taste with light (PC6; memory function). These flies were again tested 6 hours later to see if they remember to make this negative association of bitter taste with light (PC6; memory function). Pass Rate for each fly was averaged out of five trials for PC0 and PC6. Score differences between control and experimental groups were assessed using a Student t-test.
Figure 2.1 T-Maze Setup for Aversive Phototaxis Suppression Assay. (A) The overall experimental setup for the APSA experiment with the light source connected to the "lighted" falcon tube, lined with filter paper and the "dark" tube on the left (covered in foil), separated by a trap door. (B) During the training and testing phase, the fly is in the dark chamber and the trap door opened after the light is turned on in the lighted chamber.
2.8.2 – Negative Geotaxis Assay

Briefly, a group of ten age-matched female flies per genotype were placed in a vial marked with a line drawn horizontally 8 cm above the surface. The flies were tapped to the surface and given 10 s to demonstrate climbing activity as a negative geotactic response. After 10 s, the number of flies that successfully climbed above the 8 cm line was recorded. This assay was repeated 10 times and the averaged data (S.E.M.s) represented as percentages where the number of flies above the 8 cm mark was divided by the total number of flies tested within each group.

2.8.3 – Thermotolerance Assay

A thermotolerance assay was carried out as described (Gong and Golic, 2006). Briefly, 1–2-day-old flies were sexed and put into empty vials in groups of 20. The flies were given a mild heat shock in a 35 °C water bath for 30 min and immediately transferred to a 39 °C water bath. Starting from this time, the flies were examined at 10-min intervals under a light microscope, the number of paralyzed flies was counted, and then quickly returned to the 39 °C water bath. Flies were considered paralyzed if they did not move any parts of their bodies, even after the vials were tapped. The incubation at 39°C continued until all of the flies were paralyzed.
2.8.4 – Lifespan Assay

Adult flies of various genotypes were collected and sexed 24 – 48 h after eclosion. Cohorts of 200 – 300 flies were placed in groups of 25 individuals. Each group was placed in vials with no drug or with 2 mM Paraquat-supplemented food. Every day, the number of dead flies in each vial was recorded, and every third day, the flies were transferred to new vials containing fresh food with or without drug. This process was followed until all of the flies died, and the percentage of flies alive at each time point was graphed (Gong and Golic, 2006). The results were statistically tested with the Kaplan-Meier analysis with a semiparametric log-rank test (Walker et al., 2006), and the analysis was performed in Microsoft Excel.

2.9 – Immunoprecipitation Assays

For immunoprecipitation experiments, 40 2-Day old female fly heads were homogenized in lysis buffer, precleared with Protein-G beads for an hour and incubated with Protein-G beads conjugated with 10μG antibody per sample overnight at 4°C. The bead pellets were collected and washed four times with lysis buffer before eluting out the bead fraction with 4X Lamelli buffer, heating the samples at 95°C and centrifuging for 5 minutes to collect the bead eluted fraction.
2.10 – Microtubule Spin-Down Assay

To test whether NMNAT bound to intact microtubules, in vitro microtubule binding assay was performed using the Microtubule Binding Protein Spin-down Assay Kit (Cytoskeleton Inc., Denver, CO), according to manufacturer’s protocol. Briefly, this assay allows the identification of proteins that will bind to microtubules (MTs) in vitro, relying on the fact that MTs will pellet when centrifuged at 100,000Xg.

*Dm* NMNAT was cloned into pET-28b vector allowing for large-scale protein expression in bacterial culture, upon induction with imidazole. NMNAT protein was eluted into a buffer containing low salt and neutral pH with DTT. To assemble microtubules in vitro, 100µg tubulin protein was allowed to polymerize in Cushion Buffer at 35°C for 20 minutes and stabilized at room temperature with 2mM Taxol in General Tubulin Buffer. For the assay, binding reactions were set up for 30 minutes at room temperature with two positive controls (MAP: 1mg/mL; Tropomyosin: 1mg/mL), a negative control (BSA: 5mg/mL), and NMNAT (1mg/mL) with or without the pre-assembled microtubules. Using Beckman Ultraclear™ ultracentrifuge tubes (5mm P.A. Tube, Catalog# 342630, Beckman Coulter Inc., Brea, CA), the samples were placed carefully on top of Taxol supplemented Cushion Buffer. The samples were then centrifuged at 100,000Xg at room temperature for 40 minutes and the pellet fraction collected for the microtubules and the supernatant collected to test for presence of non-microtubule associated proteins. Samples were prepared in Lamelli buffer and resolved with SDS-PAGE. The polyacrylamide gel was then developed in Coomassie stain to identify specific protein bands.
Chapter 3. NMNAT Is A Stress-Responsive Protein

Recent studies in Drosophila have uncovered the protective effects of the NAD synthase NMNAT against neuronal excitotoxicity- or polyglutamine protein-induced neurodegeneration, and injury-induced axonal degeneration (MacDonald et al., 2006; Zhai et al., 2006; Zhai et al., 2008). Studies in mammalian neurons have also shown that overexpression of NMNAT protects against injury- or stress-induced axonal degeneration (Gilley and Coleman, 2010; Sasaki et al., 2009). The neuroprotective function of NMNAT is in part mediated through its chaperone activity independent of its NAD synthesis function (Zhai et al., 2008). Although the neuronal maintenance and protective effects of NMNAT have been demonstrated, it is unclear how the nmnat gene is regulated in vivo. Interestingly, it has been observed that when human Ataxin-1 with an 82 polyglutamine expansion (hATX1[82Q]) was expressed in Drosophila brain, endogenous NMNAT protein was up-regulated and recruited to the hATX1[82Q] aggregates (Zhai et al., 2008), indicating that the nmnat gene may respond to proteotoxic stress. Understanding the stress response of NMNAT and the regulatory mechanisms of nmnat gene expression will be important for the design of neuroprotective strategies.

In this chapter, the role of NMNAT in stress is investigated, showing that NMNAT is a stress protein that is transcriptionally up-regulated upon various stress conditions, including heat shock and hypoxia, and characterized the trans-
-criptional circuits involved in the regulation of neuroprotective NMNAT upon induction of stress. Furthermore, this chapter provides an in vivo mechanism for transcriptional regulation of NMNAT under stress and extend the stress response network to include a metabolic enzyme that is critical for neuronal maintenance and protection.

3.1 – NMNAT is upregulated at the protein level post acute stress

To test whether the housekeeping enzyme NMNAT is a stress protein, the effects of various stress conditions on NMNAT expression was examined in wild-type flies. Three stress paradigms were used in this study: heat shock, oxidative stress and hypoxic stress. For heat shock stress, wild-type adult flies of 2-3 days of age were subjected to two one-hour heat shocks at 37°C, with a 45-min room temperature period of acclimatization between shocks (Chomyn et al., 1979). Oxidative stress was induced by feeding flies with the free radical-producing agent 1,1’ dimethyl-4-4’-bipyridynium dichloride (Paraquat). Paraquat is a quaternary nitrogen herbicide, a very toxic substance leading to acute poisoning and death (Sittipunt, 2005). The toxicity of paraquat has been attributed to the generation of the superoxide anion leading to the synthesis of more toxic reactive oxygen species (ROS) such as hydroxyl radicals and hydrogen peroxide (Suntres, 2002). To induce acute oxidative stress, 2 day-old flies were fed media containing 5, 10 or 20 mM paraquat. Hypoxic stress was induced by exposing adult flies to 5% O₂ for various durations.
To monitor the induction of stress, heat shock protein Hsp70 was used as a positive control. As shown in Figure 3.1, heat shock, oxidative stress and acute hypoxia upregulated Hsp70 at the protein level (Figure 3.1A), while the level of actin stayed constant during stress (Figure 3.1A). Hsp70 is a heat shock protein that is not expressed under normal conditions (Kabani and Martineau, 2008); however, a low protein signal was detected at pre-stress conditions (Figure 3.1A). This is likely owing to antibody cross-reactivity to Hsc70 protein, a constitutively expressed protein (Kabani and Martineau, 2008). To test the specificity of the Hsp70 antibody, brain lysates from non-stressed Hsp70 null flies (Df(3R)Hsp70A, Df(3R)Hsp70B) that lack all 12 copies of the Hsp70 gene, were probed with the same Hsp70 antibody which detected a low signal at 70 kDa (Figure 3.2), suggesting the antibody has slight cross-reactivity to Hsc70 and the low level signals detected at pre-stress conditions correspond to Hsc70.

The level of NMNAT expression was also determined at the protein level by quantitative western blotting using a NMNAT-specific antibody (Zhai et al., 2006). Interestingly, NMNAT protein (Figure 3.1A) showed a similar trend of upregulation, as did Hsp70 under stress conditions. Specifically, heat shock at 37°C induced a 3-fold increase in protein level between 8-12 hours post-heat shock (Figure 3.1A). Oxidative stress induced by paraquat also caused a dose-dependent induction in NMNAT protein level where a 3.3-fold increase was seen in flies fed with 20 mM paraquat for 72 hours (Figure 3.1A). Acute exposure to
Figure 3.1 NMNAT is induced upon stress. (A) Heat shock, oxidative stress and acute hypoxia each upregulated NMNAT protein level in vivo. Wild-type flies were used in these stress paradigms. Heat stress consisted of shocking flies in a 37°C water bath for one hour, twice, with a 30-min period of room temperature acclimatization between treatments. Oxidative stress was induced by feeding flies 5, 10 or 20 mM Paraquat. Hypoxic stress was induced by exposing adult flies to 5% O₂ for various durations. Actin was used as a loading control and Hsp70 as a positive control for these stress conditions. (B-C) nmnat (B) and hsp70 (C) transcription levels were significantly increased post-stress as measured by quantitative real-time RT-PCR. rp49 was used as a housekeeping control for normalization. Values are expressed as mean ± S.E.M. n≥4. Significance level was established by one way ANOVA post-hoc Scheffe test. *p<0.05; **p<0.001.
hypoxia (5% O₂) for 3 hours resulted in a 3.1-fold upregulation in NMNAT level (Figure 3.1A). Note that additional protein bands above the NMNAT band (29 kDa, arrowhead in Fig. 3.1A) were detected in protein samples from heat shock- and hypoxia-treated groups (Figure 3.1A). Since the antibody has been shown to specifically recognize NMNAT (29 kDa) (Zhai et al., 2006) and the nature of these addition bands was unclear, only the specific 29 kDa NMNAT band was included in this study. These results show that NMNAT was upregulated under heat stress, hypoxia, or oxidative stress in a time or dose-dependent manner similar to the stress protein Hsp70 (Fig. 3.1) as reported previously (Banerji et al., 1984; Lee, 1992; Lee and Corry, 1998).

3.2 – NMNAT is transcriptionally upregulated by acute stress paradigms

To see if the upregulation of NMNAT was consistent at the mRNA level, quantitative real-time PCR was used to investigate NMNAT and HSP70 transcript levels in wild type flies using the same stress paradigms used earlier. As seen in Figure 3.1C, Hsp70 was upregulated transcriptionally by all three stress
conditions. Moreover, since Hsp70 is almost not present under non-stressed conditions, a much higher fold induction is observed transcriptionally, with Hsp70 fold change reaching up to 100-120 within 3 hours post heat shock. A modest upregulation in NMNAT mRNA levels is also observed upon all three stress conditions (Figure 3.1B). In fact, there is a 3-fold induction of NMNAT levels by 3 hours post heat shock, similar to the fold change observed after 3 hours of hypoxia (Figure 3.1B). In fact, the results in this study suggest that NMNAT may be transcriptionally upregulated under a variety of stress conditions.

3.3 – NMNAT is essential for stress tolerance in Drosophila

To directly determine the role of NMNAT during acute stress, the requirement of NMNAT was examined for thermotolerance in adult flies. Wild-type flies are able to tolerate high temperatures (e.g. 39°C) when pre-conditioned with a mild heat shock (e.g. 35°C for 30 minutes) to stimulate the synthesis of HSPs (Gong and Golic, 2006). In contrast, flies with reduced levels of HSPs such as Hsp70 have significantly reduced thermotolerance (Gong and Golic, 2006). The thermotolerance of flies that were heterozygous for either HSP70 (Df3R Hsp70A,Df3RHsp70B/TM6; Figure 3.3 A-3) lacking 6 of the 12 copies of Hsp70 or NMNAT (nmnat +/-; Figure 3.3 A-2) or both HSP70 and NMNAT (Df3R Hsp70A,Df3RHsp70B/nmnat; Figure 3.3 A-1) or HSF (Hsf3/CyO; Figure 3.3A-8), were investigated. In addition, thermotolerance was also studied in hsf4 flies (Figure 3.3A-4) that fail to induce heat shock response at 37°C. In addition, the
Figure 3.3 NMNAT is required for stress tolerance in Drosophila. (A) Flies of different genotypes were preconditioned for 30 min in a 35 °C water bath and then exposed to 39 °C. The time until paralysis onset was measured. The values are expressed as the means±S.E. of 8–10 experiments, where each experiment consisted of 20 flies. (B) NMNAT expression significantly improved survival in flies exposed to Paraquat-induced oxidative stress. The flies were maintained on either a 0 or 2 mM Paraquat diet (in 5% sucrose, 1% agar) from 1 day post-eclosion. The flies were transferred to fresh food every 2 days. The survival rates were recorded for each genotype. Wild-type flies had severely compromised lifespan upon 2 mM Paraquat exposure. NMNAT heterozygous flies had further compromised lifespans. Overexpressing either Drosophila or human NMNAT in the CNS with elav-Gal4 driver significantly prolonged the lifespan of flies upon exposure to Paraquat.
effect of NMNAT overexpression in the brain (Figure 3.3A-7) or globally (tubulin-GAL4>NMNAT; Figure 3.3A-9) on stress-tolerance was compared to wild-type (Figure 3.3A-5), NMNAT overexpression in Hsp70 heterozygous background (elav-GAL4, Df(3R)Hsp70A, Df(3R)Hsp70B/UAS-NMNAT; Figure 3.1.3A-6), or NMNAT overexpression in Hsf3 background (Hsf3/elav-GAL4; UAS-NMNAT; Figure 3.3A-8). As shown in Figure 3.3A, heterozygous nmnat+/- flies showed a much reduced thermotolerance, as indicated by the shortened time to paralysis upon exposure to high temperature (39°C), whereas overexpressing NMNAT ubiquitously with tubulin-GAL4, or in the nervous system using elav-GAL4, increased thermotolerance. It is important to note that loss of one copy of nmnat caused a more severe reduction of thermotolerance compared to Hsp70 heterozygous flies, suggesting the essential role of NMNAT in thermotolerance. Moreover, thermotolerance is further reduced with loss of one copy of NMNAT and 6 of the 12 copies of HSP70. However, loss of one copy of HSF did not impart significant differences to thermotolerance, showing that there was enough HSF present to cause transcriptional upregulation of protective factors upon heat stress. Conversely, loss of heat shock response in the hsf4 flies rendered flies intolerant to thermotolerance. These results suggest that NMNAT functions physiologically as a stress protein and that its expression levels are important determinants of stress tolerance in flies.

To understand the role of NMNAT in chronic oxidative stress response, alterations in lifespan of flies under paraquat-induced oxidative stress was
investigated. Increased paraquat resistance has been correlated with increased lifespan in long-lived mutants of *C. elegans*, *Drosophila* and mouse (Lin et al., 1998; Migliaccio et al., 1999; Yanase et al., 2002). Hence, the resistance to dietary paraquat was measured to determine the role of NMNAT in resistance to oxidative stress and prolonging lifespan. Newly eclosed adult flies were reared on 2 mM paraquat to measure lifespan. As shown in Figure 3.3B, wild-type flies had severely compromised lifespan; however, overexpressing either wild type or enzyme-inactive NMNAT with Actin-Gal4 driver significantly prolonged lifespan of flies raised on paraquat. Furthermore, *nmnat* +/- heterozygous flies displayed a shortened lifespan compared to wild-type, suggesting that NMNAT contributes to the organismal resistance to oxidative stress. These results support the role of NMNAT as a stress protein and that its expression levels are important determinants of oxidative stress tolerance and lifespan in flies.

3.4 – Identification of putative binding sites for stress-related transcription factors in Dm *nmnat* promoter

To understand the transcriptional regulation of *nmnat* gene under stress, the promoter region of the Drosophila *nmnat* gene was analyzed in detail to locate transcription factor binding sites using the bioinformatics program MatInspector (Cartharius et al., 2005). MatInspector is a software tool within the Genomatix Portal software that utilizes a large library of matrix descriptions for transcription factor binding sites to locate matches in DNA sequences. It assigns a quality rating to matches and thus allows quality-based filtering and selection of matches.
(Cartharius et al., 2005). Using MatInspector, putative binding elements for both Hsf (Figure 3.4A,C) and Hif-1α (Figure 3.4B,D) were identified within the promoter region of *nmnat*, providing *in-silico* evidence of the possible regulation of *nmnat* via transcription factors implicated in various stress conditions such as heat shock and oxidative stress. Furthermore, these possible transcription factor binding sites are conserved between various isoforms of human, mouse and *Drosophila nmnat*. In fact, two heat shock elements (HSE) were identified in the *nmnat* promoter region: HSED for HSE distal and HSEP for HSE proximal, referring to their relative distance to the transcription start site (TSS). HSED

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**Figure 3.4 In silico prediction of putative transcription binding sites in Dm nmnat promoter region.** (A) Putative HSE (green) in *Dm nmnat* and *Hs nmnat1-3* promoter regions. (B) Putative HRE (red) in the in *Dm nmnat* and *Hs nmnat1-3* promoter regions (C-D) Sequence analysis of putative transcription factor (HSF and Hif1-α) binding elements (HSE and HRE) for hsp70, nmnat and hsf.
consists of a single pentameric nGAAn element while HSEp consists of three contiguous inverted repeats of the pentameric element, comparable to those found in the proximal promoter region of HSP genes (Schiller et al., 1988; Xiao and Lis, 1988) (Figure 3.4C). As HSF trimerization is required for its binding to HSE (Abravaya, 1992), the HSEd in nmnat promoter with a single pentameric nGAAn is less likely than HSEp to be functional. At the same time, the hypoxia response element (HRE) in nmnat promoter is comprised of a single conserved “ACGT” core sequence (Figure 3.4D).

3.5 – Changes in Hsf or Hif1-α in vivo affect the level of NMNAT upon stress

To examine whether Drosophila Hsf directly regulates the transcription of nmnat upon heat shock, the expression and induction of nmnat upon heat shock was measured in Hsf gain- or loss-of-function flies. For Hsf gain-of-function, a pan-neuronal driver elav-GAL4 driver was used to overexpress UAS-HSF in the nervous system. For Hsf loss-of-function, a temperature-sensitive mutant allele hsf4 was used, where HSF is inactivated at 37°C and unable to mediate transcriptional activation upon heat shock (Jedlicka et al., 1997). As shown in Figure 3.5, in HSF-overexpressing flies (HSF OE), NMNAT protein was induced to a higher level in the brain upon heat shock, and both NMNAT and Hsp70 proteins were present at a moderate level prior to heat shock (Figure 3.5A middle column), while in HSF loss-of-function flies (hsf4) the induction of both Hsp70 and NMNAT upon heat shock was diminished (Figure 3.5A, right column). Note that
NMNAT levels in HSF mutant or overexpressing flies upon heat shock. HSF overexpression using elav-GAL4 induced a stronger induction of both Hsp70 and NMNAT upon heat shock. Loss of HSF in hsf4-mutant flies diminished the induction of both Hsp70 and NMNAT upon heat shock. (B) NMNAT levels in Sima mutant or overexpressing flies upon hypoxia. Sima overexpression in the nervous system enhanced upregulation of both NMNAT and Hsp70 upon hypoxia. Loss of one copy of Sima failed to upregulate NMNAT or Hsp70 upon hypoxia. However, the heat shock pathway was intact in Sima+/- mutant flies, as both NMNAT and Hsp70 were upregulated following heat shock. (C) Changes in nmnat mRNA levels in HSF mutants upon heat shock, normalized to rp49. HSF overexpression enhanced the upregulation of nmnat transcript while loss of HSF diminished upregulation post-heat shock. (D) Changes in nmnat transcription during hypoxia in Sima heterozygous or overexpressing flies, normalized to rp49. Sima overexpression strongly induced nmnat transcription compared to wild-type flies, while loss of one copy of Sima abolished transcriptional upregulation of nmnat upon hypoxia.
the Hsp70 antibody detects a faint band of the appropriate size in $hsl^d$ flies and in wild-type flies under no stress condition (Figure 3.5A), which is likely due to antibody cross-reactivity to Hsc70 protein (Figure 3.2).

The effect of HSF on $nmnat$ expression was also evident at the transcript level, as measured by quantitative realtime PCR. As shown in Figure 3.5C, the number of $nmnat$ transcripts was upregulated upon heat shock in wild type ($yw$) flies, and this upregulation upon heat shock was enhanced in HSF-overexpressing (HSF OE) flies and was diminished in loss-of-HSF ($hsl^d$) flies. These results suggest that heat shock transcription factor HSF is required and responsible for the upregulation of NMNAT under heat shock.

In addition to heat shock, hypoxic stress also induced $nmnat$ gene expression in wild-type flies (Figure 3.1). Next, the requirement for HIF1α for the upregulation of NMNAT under hypoxic stress was also examined. The expression and induction of $nmnat$ upon hypoxia (0.5% $O_2$) in HIF1α gain- or loss-of-function flies was measured here. For loss of function, a mutant allele of Sima (Lavista-Llanos et al., 2002), the Drosophila homologue of HIF1α, was used (Bacon et al., 1998). For gain of function, the pan-neuronal driver $elav-GAL4$ driver overexpressed $UAS-Sima$ in the nervous system. When Sima was overexpressed, moderate levels of NMNAT and Hsp70 protein and mRNA levels were already observed in fly brain extracts prior to hypoxia and further enhanced the upregulation of NMNAT upon hypoxic stress.
(Figure 3.5B and 3.5D). In contrast, flies heterozygous for Sima+/- failed to upregulate NMNAT or Hsp70 upon hypoxia. The diminished upregulation is specific for the hypoxic stress pathway, as both NMNAT and Hsp70 were upregulated in Sima+/- mutant flies following heat shock stress, suggesting that the heat shock pathway was intact in these flies (Figure 3.6). These results suggest that hypoxia transcription factor HIF1α is required to induce the upregulation of NMNAT under hypoxia.

**Figure 3.6 NMNAT is not upregulated in hsf4 mutant flies post-hypoxia.** (A) Temperature-sensitive hsf4 mutant flies that lack initiation of a heat shock response at 37°C were maintained in hypoxic conditions (5% O2) for 2 to 12 hours. Hypoxia failed to induce either NMNAT or Hsp70 in hsf4 mutant flies, demonstrating that HSF is a key transcription factor for regulating the levels of NMNAT during hypoxia. Actin was used as a loading control. (B) Levels of NMNAT and Hsp70 were quantified by densitometry and normalized to actin levels.

In order to assess stress responses at the cellular level, mosaic analyses was used to examine the effects of HSF or Sima on NMNAT expression. Using a photoreceptor-specific driver GMR-Gal4 (Hay et al., 1997), HSF or Sima was overexpressed together with green fluorescent protein (GFP) specifically in the
Overexpression of HSF or Sima upregulates NMNAT upon stress in the eye disc. GFP (A-C), HSF-eGFP (D-E), or Sima (F-G) was expressed in the L3 larval eye imaginal disc with a GMR-Gal4 driver. L3 larvae were subjected to heat shock, hypoxia or normal condition and eye discs were dissected and stained for NMNAT (red), Hsp70 (magenta) and DAPI (blue). (A-C) Heat shock or hypoxia caused an overall induction of NMNAT and Hsp70 throughout the eye disc. (D-E) HSF-eGFP overexpression caused NMNAT and Hsp70 upregulation in the GMR-expressing region upon heat shock. (F-G) Sima overexpression caused an increase of both NMNAT and Hsp70 levels after hypoxia in the GMR-expressing region.
posterior half of the eye primordium in third instar (L3) larval eye imaginal discs. As shown in Figure 3.7, in the eye primordium cells demarcated by GFP where either HSF or Sima was overexpressed, a greater induction of NMNAT was observed when L3 larvae were subjected upon either heat shock or hypoxia, respectively. Overexpressing either transcription factor under normal conditions did not induce the expression of NMNAT or Hsp70 (Figure 3.7 A2-3, D2-3, F2-3) as both these factors are activated by stress (Jaakkola et al., 2001; Lerman and Feder, 2001). These results suggest that HSF or Sima induces NMNAT upregulation under stress in a cell-autonomous manner.

3.6 – Hsf binds to nmnat promoter to induce transcription during acute stress

The work so far suggested that the stress transcription factors Hsf and HIF1-α/Sima can regulate nmnat gene expression. To understand the molecular mechanism underlying the transcriptional regulation, it was essential to examine whether Hsf or Sima could directly bind to the promoter region of nmnat and induce transcription upon stress, and whether the predicted consensus HSEs and HRE are functional. In silico analysis has previously revealed two possible HSF binding regions named HSEd for HSE distal and HSEp for HSE proximal referring to their relative distance to the transcription start site (TSS) (Figure 3.4).

Using cultured Drosophila S2 cells, chromatin immunoprecipitation (ChIP) assay was conducted to detect promoter occupancy for both Hsf and Sima
transcription factors. As shown in Figure 3.8A, upon heat shock, Hsf bound to both HSE sites, with stronger binding at the proximal HSE site. A similar level of HSF occupancy was also observed for the HSE-containing region of *Drosophila hsp70* (Boehm et al., 2003). To carry out the ChIP analysis of the HRE element, S2 cells were transfected with HA-tagged HIF1-α (HA-Sima) and its transcriptional co-factor HA-Tango (Lavista-Llanos et al., 2002; Romero et al., 2008) and subjected S2 cells to hypoxia induced by treatment of 100 µM deferoxamine mesylate (DFO) for 16 hours (Dekanty et al., 2005). When chromatin was precipitated with anti-HA antibody, promoter occupancy of Sima

![Figure 3.8 HSF binds to the predicted HSE in the promoter region of nmnat.](image)

(A) ChIP analysis of nmnat HSE. S2 cells were transfected with constructs containing two HSEs (HSEp+d) or HSEp and subjected to heat shock. ChIP was carried out using antibody against HSF. Occupancy of both HSEs by HSF was observed upon heat shock, with a greater enrichment at HSEp. The HSE-containing region of hsp70 was used as a positive control, and the promoter region of rp49 was used as a negative control. (B) ChIP analysis of nmnat HRE. S2 cells were transfected with constructs containing HRE and HA-tagged Sima and subjected to hypoxia induced by 100 µM DFO. ChIP analysis was carried out using anti-HA antibody. No occupancy of HRE by Sima was observed during hypoxia. The promoter region of *Drosophila* Hph was used as a positive control, and the promoter region of actin was used as a negative control. Sima occupancy was detected at an HRE in the second intronic region of hsf upon hypoxia. *Pol*, polymerase; *IP*, immunoprecipitation; *Ab*, antibody.

at the predicted HRE-containing region of the *nmnat* gene was not detected (Figure 3.8B), although Sima occupancy was detected in the genomic region
containing an HRE of a known HIF1α target *Hph* (Baird et al., 2006). Interestingly, the binding of Sima to an HRE in the second intronic region of *hsf* gene was also detected upon hypoxia (Figure 3.8B), confirming the previous report that HSF is transcriptionally upregulated by HIF1α during hypoxia (Baird et al., 2006). Collectively, these results indicate that heat shock factor HSF can bind to the heat shock elements in *nmmat* promoter, preferentially to the proximal HSE upon heat shock. However, hypoxia-inducible factor Sima/HIF1α does not bind to *nmmat* promoter directly upon hypoxia. It is likely that Sima upregulates *nmmat* transcription upon hypoxia indirectly through upregulation of HSF, which in turn directly binds to *nmmat* promoter and initiates transcription.

To further examine the functionality of these transcription factor binding sites, a dual luciferase reporter assay (Promega) was employed to measure the transcriptional activity of luciferase reporter constructs that contain either the entire *nmmat* promoter region (*nmmat*Pro), or regions containing wild-type or mutant HSEs (HSEp+d or mHSE) (Figure 3.9). S2 cells that were transfected with constructs containing two HSEs (HSEp+d) or the proximal HSE (HSEp) showed a significant induction of luciferase activity post-heat shock (Figure 3.9B). When two of the residues in the second pentameric repeat in HSEp were mutated (mHSEp) to abolish the binding of HSF trimer, the heat shock-induced luciferase activity was diminished. In contrast, mutating HSEd had little effect on luciferase induction post-heat shock, suggesting that the distal HSE site was nonfunctional. These results suggest that the proximal heat shock element
(HSEp) is necessary and sufficient to mediate HSF binding and nmnat induction upon heat shock.

When similar analysis was carried out for hypoxic stress, upon hypoxia induction by 100 µM DFO, S2 cells (immortalized Drosophila hemolymph cells) transfected with a luciferase construct containing the full length promoter (nmnat<sup>Pro</sup>) showed induction of luciferase activity to a level that was comparable to that of positive control cells transfected with a construct containing a functional HRE in the promoter of mouse VEGF gene (Dougherty et al., 2008) (Figure 3.9C), indicating the transcriptional activation of nmnat upon hypoxia. However, this transcriptional activation was abolished when both HSEs sites were mutated in this construct, leaving only intact HRE (HRE+mHSEs), and proximal HSE alone was necessary and sufficient to restore the transcriptional activity upon hypoxia (Figure 3.9C). Collectively, these results show that the predicted HRE element in the nmnat promoter region was nonfunctional and instead, the integrity of the proximal HSE was required for nmnat upregulation upon hypoxia. The luciferase data therefore indicate that the proximal HSE is the key promoter element mediating stress transcription factor binding under both heat shock and hypoxic conditions.
Figure 3.9 The proximal HSE in nmnat promoter region is necessary and sufficient for transcription induction under heat shock and hypoxia. (A) Analysis of the consensus binding elements for both HSF and Sima in *Drosophila nmnat* promoter. (B and C) The functionality of nmnat promoter elements was tested using dual luciferase reporter assay. The promoter region of nmnat with various lengths and mutations in A was cloned into a minimal firefly luciferase vector (pGL4). Renilla luciferase was used to normalize for transfection efficiency. S2 cells were transfected with luciferase constructs and treated with heat shock (B) or hypoxia (C). Constructs containing two HSEs (HSEp+d) or the proximal HSE (HSEp) showed a significant induction of luciferase activity post-heat shock (B). mHSEp diminished the heat shock-induced luciferase activity, but mHSEd had little effect on luciferase induction post-heat shock (B). Upon DFO-induced hypoxia, nmnatPro and mHSEd showed induction of luciferase activity similar to positive control, whereas HRE+mHSEs with only the HRE intact failed to induce luciferase activity upon hypoxia (C).
To further evaluate the cellular requirement for HSF in NMNAT stress response in vivo, mosaic analyses was used again to examine the cellular effects of either gain or loss of function of HSF under stress conditions. Using bbg-Gal4 driver (Kim et al., 2006), HSF-eGFP was overexpressed in a patch of cells in the third instar (L3) larval wing imaginal disc that will become the future wing margin (Kim et al., 2006). As shown in Figure 3.10A–L, the GFP signal demarcates cells overexpressing HSF, where a greater induction of NMNAT was observed upon either heat shock or hypoxia (Figure 3.10). In contrast, when HSF was down-regulated in these cells using HSF-RNAi driven by bbg-GAL4, a reduction in NMNAT was observed in the cells with no HSF, compared with the surrounding wild-type cells upon both heat and hypoxic stress (Figure 3.10M–X). These results further suggest that HSF is required for the induction of NMNAT under both heat shock and hypoxic stress in a cell-autonomous manner.

The in vivo analysis on nmnat promoter and gene expression suggest the following model for nmnat transcriptional regulation upon stress: upon heat shock, HSF directly binds to nmnat proximal HSE and induces transcription; upon hypoxia, HIF1α/Sima binds to the HRE element in the HSF intronic region and up-regulates HSF (Baird et al., 2006), which subsequently induces transcription via nmnat HSE.
Figure 3.10 HSF regulates the expression of NMNAT upon stress. (A-L) Hsf-eGFP was overexpressed in the wing margin of the L3 wing imaginal disc with bbg-GAL4. The wing discs were dissected from L3 larvae at 12 hours after no stress (A-D), heat shock (E-H), or hypoxia (1% O$_2$) (I-L), and stained for NMNAT (red), HSF (magenta) and DAPI (blue). GFP demarcated Hsf overexpression. (M-X) hsf-RNAi was overexpressed in the L3 imaginal disc with bbg-GAL4. The wing discs were dissected from L3 larvae at 12 hours after no stress (M-P), heat shock (Q-T), or hypoxia (1% O$_2$) (U-X) and stained for NMNAT (red), HSF (magenta) and DAPI (blue). Scale bar: 20 µm.
3.7 – Hsf level is increased during hypoxia and is regulated by Hif1-α

The lack of HIF1α binding to the nmnat promoter would suggest that during hypoxia, nmnat is transcriptionally upregulated by Hsf binding. This is absolutely plausible since ChIP data in Figure 3.8B already show in agreement with Baird et al. (2006) that HIF1α binds to the HRE in the second intron of hsf gene. In fact, if this binding is functional, it would result in an upregulation of HSF at the protein level in hypoxia (Figure 3.11). In wild type flies, heat shock does not induce an upregulation of Hsf, although both NMNAT and Hsp70 are upregulated (Figure 3.11A, Lanes 1-5). However, hypoxia causes a steady increase in Hsf protein, consistent with an increase in NMNAT and HSP70 (Figure 3.11A, Lanes 6-9).

Figure 3.11 HSF is up-regulated upon hypoxia in a HIF1α-dependent manner. (A) The levels of HSF, NMNAT, and Hsp70 upon hypoxia in wild-type and Sima-overexpressing flies upon hypoxia and heat shock. Heat shock induced a concomitant up-regulation of NMNAT and Hsp70 without affecting HSF levels (lanes 1–5). Hypoxia induced a concomitant up-regulation of HSF, NMNAT, and Hsp70 (lanes 6–13). In Sima overexpression flies, the up-regulation of all three proteins HSF, NMNAT, and Hsp70 was more robust (lanes 10–13). (B) NMNAT levels did not affect HSF or Hsp70 levels. The protein level of NMNAT, Hsp70, HSF, and actin were determined in wild-type (+/+), NMNAT heterozygous (+/-), and NMNAT-OE (OE) flies by Western blot analysis. No difference was detected in the levels of Hsp70, HSF, or actin.
More interestingly, this upregulation in HSF levels is dependent on HIF1α levels, as overexpression of HIF1α pan-neuronally causes a more robust induction in HSF levels, consistent with its targets, NMNAT and HSP70 (Figure 3.11A, Lanes 10-13). To exclude the possibility that NMNAT levels might have an effect on HSF, the levels of Hsf were investigated in flies lacking a copy of NMNAT and compared this to levels in wildtype and pan-neuronally overexpressing NMNAT flies. As shown in Figure 3.11B, changes in NMNAT levels have no effect on HSF or HSP70 levels.
Chapter 4. NMNAT2 Downregulation Precedes Disease Phenotype In P301L Tau Transgenic Mouse Model

Tauopathies comprise of several neurodegenerative diseases such as Pick’s disease and fronto-temporal dementia with parkinsonism linked to chromosome 17 (FTDP-17; reviewed in (Lee et al., 2001)). The causative agent in tauopathies is hyperphosphorylated microtubule-associated protein tau which makes up intracellular neurofibrillary tangles (NFTs). Several tau mutations increase susceptibility to FTDP-17 have been identified (Bugiani, 1999; Goedert and Jakes, 2005; Hutton, 2000; Poorkaj et al., 2001; Sperfeld et al., 1999; Spillantini and Goedert, 1998) and transgenic mice expressing human wildtype or mutated tau showed NFT formation and neuronal loss, implying a causal relationship between tau over-expression and neurodegeneration (e.g., (Andorfer et al., 2003; Gotz et al., 2001; Lewis et al., 2000; Yoshiyama et al., 2007). Although there is evidence for the presence of activated caspase and morphological features of necrosis, autophagy, and abnormal cell cycle events (Andorfer et al., 2005; Andorfer et al., 2003; de Calignon et al., 2010; Gamblin et al., 2003; Lin et al., 2003; Rissman et al., 2004) the precise mechanism of cell death in tauopathies remains unknown.

In previous studies, loss of nmnat in the Drosophila eye have been shown to cause rapid neuronal degeneration (Zhai et al., 2006). Three mammalian homologs, NMNAT1-3, have been identified (Allegrini et al., 2002; Raffaelli et al., 2002; Zhang et al., 2003), with NMNAT2 being the most labile of the three iso-
-forms with a half-life of less than four hours (Gilley and Coleman). Knocking down endogenous NMNAT2 levels in cultured mouse neurons induced Wallerian-like degeneration, suggesting a role for NMNAT2 in maintaining axonal health in the peripheral nervous system (Gilley and Coleman). Interestingly, several gene-array studies found that NMNAT2 levels were reduced in brain specimens from patients with Alzheimer’s disease (AD; https://www.nextbio.com). Over-expression of NMNAT, on the other hand, provides neuroprotection against several degenerative conditions in Drosophila (Zhai et al., 2006; Zhai et al., 2008b). In mammals, over-expressing NMNAT isoforms have delayed Wallerian degeneration (Araki et al., 2004; Press and Milbrandt, 2008; Sasaki and Milbrandt; Sasaki et al., 2009; Wang et al., 2005; Yahata et al., 2009).

To explore the role of NMNAT in neurodegeneration found in tauopathies, the expression levels of NMNAT1-2, two homologs present in brain, were investigated in rTg4510 mice at various ages. In rTg4510 mice (Ramsden et al., 2005; Santacruz et al., 2005), tau with the mis-sense mutation P301L, found in some FTDP-17 cases (Hutton et al., 1998), is over-expressed in the forebrain. Here, mutant Tau with the mis-sense mutation P301L, found in some FTDP-17 cases (Hutton et al., 1998), was over-expressed in the forebrain with the CaMKII promoter. This allowed for expression around postnatal day seven and peaking around one month of age (Ramsden et al., 2005; Santacruz et al., 2005). From two and half months onward, hyperphosphorylated tau protein and neuronal loss
can be detected in the forebrain of these mice (Ramsden et al., 2005; Santacruz et al., 2005; Spires et al., 2006). Cognitive impairment in spatial reference memory and synaptic dysfunction in hippocampal CA1 pyramidal neurons are apparent at four and half months of age (Hoover et al., 2010). Significant neurodegeneration is observed after five months of age, being most apparent in the hippocampal CA1 area (Ramsden et al., 2005; Santacruz et al., 2005; Spires et al., 2006). Noticeable cortical thinning in rTg4510 mice occurs between four and nine months of age and neurofibrillar tangle formation and gliosis are often observed around nine months in these animals.

In rTg4510 mice (Ramsden et al., 2005; Santacruz et al., 2005), the age-dependent loss of neurons and cognitive dysfunction in rTg4510 mice closely mimics the clinical features of tauopathy. The most interesting finding here was that the levels of phospho-CREB and NMNAT2 were decreased by tau_{P301L} over-expression prior to the onset of neurodegenerations in rTg4510 mice. The reduction in \textit{nmanat2} transcription in rTg4510 mice likely resulted from reduced CREB activity.

4.1 NMNAT2 is downregulated in rTg4510 brain prior to neurodegeneration

To examine the level of NMNAT2 in brain tissue over-expressing tau_{P301L}, Western blot analysis was conducted to quantitatively compare NMNAT2 protein abundance between rTg4510 mice and their littermate controls. Lysates of cortex, hippocampus, and cerebellum were prepared from rTg4510 mice and
their age-matched littermate controls at 2 weeks, one month, two months, and
seven months of age (Figure 4.1, n = 3-7 for each age group and each genotype; Table 4.1). Cerebella, where tauP301L was not over-expressed, were used as an internal control for NMNAT2 expression. Indeed, no change in the expression of cerebellar NMNAT2 was found at any of the ages examined (Figure 4A, D). Strikingly, the level of NMNAT2 in the cortex and hippocampi of rTg4510 mice was often reduced to less than 60% of the level found in their age-matched controls at one, two, and seven months of age (Figure 4.1 A-C; Table 4.1). The reduced NMNAT2 levels in rTg4510 cortex and hippocampus at one month of age coincide with the time when tauP301L over-expression begins to reach its plateau. At this age, neuronal loss was not yet observed. Thus, the decrease in cortical NMNAT2 coincides with tauP301L over-expression and precedes significant neuronal loss. The expression level of NMNAT1, another NMNAT isoform expressed in the brain (http://mouse.brain-map.org/brain/Nmnat1.html) was not altered in the cortex, hippocampus, or cerebellum of two-month-old rTg4510 mice (Figure 4.1A; Table 4.1).

To further investigate a potential reduction in neuronal population or increased gliosis in rTg4510 mice, the levels of neurofilament-medium (NF-M) and glial fibrillary acidic protein (GFAP) in rTg4510 and control mice were measured at 2 weeks, one month, and two months of ages. No alterations in the levels of actin, NF-M or GFAP were observed in rTg4510 mice at any of these time points in the cerebellum, cortex, or hippocampus (Figure 4A; Table 4.1).
These data are consistent with the immunohistochemical results from previous studies (Ramsden et al., 2005; Santacruz et al., 2005; Spires et al., 2006). These studies found that there were no gross abnormalities in the glial and neuronal populations in these brain areas of rTg4510 mice up to two months of age.

**Figure 4.1 NMNAT2 protein levels were down-regulated in the forebrain of rTg4510 mice.** (A) Western blots show NMNAT2, NMNAT1, NF-M, GFAP, actin, and hTau immunoreactivity in the cortex, hippocampus, and cerebellum of one-month-old control and rTg4510 mice (abbreviated as Tau mice). (B-D) Normalized ratios of NMNAT2/actin in the cortex (B), hippocampus (C), and cerebellum (D) of control and Tau mice at 2 weeks, 1, 2 and 7 months of age. Data are presented as percentages of their age-matched controls and are plotted as mean ± S.E.M. (for one-month-old, n = 10 for each genotype; for other age groups, n=3 for each genotype group; and * P < 0.05, ** P < 0.01, *** P < 0.001).

Using quantitative real-time RT-PCR analysis, any effects on the level of nmnat2 mRNA from tauP30L over-expression were further investigated. Specific brain areas, including the cortex, hippocampus, and cerebellum from rTg4510
Table 4.1 Quantitative analyses of Western blot. Normalized values of NMNAT2/actin, GFAP/actin, NF-M/actin, NMNAT1/actin and pCREB(Ser133)/CREB are presented as means ± S.E.M. P values were derived by student-t-test.

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<th>Cerebellum</th>
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<th>Hippocampus</th>
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<td></td>
<td>P value</td>
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<tr>
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<td>GFAP</td>
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<td>NMNAT1</td>
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<td>1 months</td>
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Table 4.2 Fold differences of nmnat2 transcript levels in rTg4510 mice compared with control mice as determined by qPCR. Values are presented as means ± S.E.M.

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<td>mean ± S.E.M.</td>
<td>t-test P values</td>
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<td>2 weeks</td>
<td>1.52 ± 0.02</td>
<td>P = 0.002</td>
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<td>P = 0.128</td>
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mice and their littermate controls at two weeks, one month, two months, and seven months of age were analyzed (n = 3 for each age group and each genotype) nmnat2 expression in rTg4510 mice was significantly down regulated at one month, two months and seven months of age in the cortex and at two months and seven months of age in the hippocampus (Figure 4.2; Table 2). No down-regulation of nmnat2 expression was seen in the cerebellum. Thus, the

![Graph showing nmnat2 expression levels in different brain regions over time.](image)

**Figure 4.2 nmnat2 transcription was down-regulated in rTg4510 forebrains.** Real-time qPCR analyses for nmnat2 mRNA levels in the cerebellum, cortex, and hippocampus of Tau mice relative to their littermate controls at 2 weeks, 1, 2 and 7 months of age. nmnat2 transcript levels were normalized to the level of the house keeping gene gapdh. Fold differences (Tau/control) are calculated with the standard 2-ΔΔCt method (see Materials and Methods) as 2(power, -ΔΔCt) and plotted as mean ± S.E.M (* P < 0.05, ** P < 0.01, *** P < 0.001).

decrease observed in NMNAT2 protein in rTg4510 mice was consistent at the transcript level in the cortex at 1 month of age and older and in the hippocampus
at two months of age and older. The specific reduction of \textit{nmnat2} expression in regions where \textit{tau}_{P301L} was over-expressed and the correlation between the time when \textit{nmnat2} levels are down-regulated and when \textit{tau}_{P301L} over-expression peaks suggest that the pathological down regulation of \textit{nmnat2} transcription is a direct consequence of \textit{tau}_{P301L} expression.

4.2 \textit{CREB activity is reduced in rTg4510 forebrains before cognitive dysfunction}

The cAMP-response element binding protein (CREB) regulates gene transcription relevant to specific cognitive tasks and is critical for synaptic plasticity and learning/memory (reviewed in (Lee and Silva, 2009)). When neurons are appropriately activated, CREB is phosphorylated at Ser133 (pCREB). pCREB then activates the transcription of several immediate early response genes (Mayr and Montminy, 2001; Sheng et al., 1991). The expression of CREB-binding protein (CBP) and many CREB downstream targets, for example BDNF and cFOS are altered in models of AD (Dickey et al., 2003; Ma et al., 2007; Palop et al., 2005; Saura et al., 2004; Tong et al., 2001; Vitolo et al., 2002). Interestingly, the abundance of pCREB (Ser133), but not the total level of CREB, is significantly reduced in 3xTg-AD mice (Caccamo et al.), transgenic mice harboring three mutated genes found in familiar AD: ß-amyloid precursor protein (ßAPPswe), presenilin-1 (PS1M146V), and tau$_{P301}$ (Espana et al.; Oddo et al., 2003a; Oddo et al., 2003b). Memory deficits and synaptic dysfunction in
rTg4510 mice are first detected at four and half months of age (Hoover et al.; Ramsden et al., 2005).

To determine whether CREB activity was reduced in the forebrain of rTg4510 mice when NMNAT2 level was reduced, the levels of pCREB(Ser133) and total CREB were in the brains of two-month-old rTg4510 (n = 5) mice and their

Figure 4.3 pCREB levels are reduced in rTg4510 forebrains. (A) Western blots show a reduction in the level of pCREB(Ser133), but not total CREB, in 2-month-old rTg4510 cortex and hippocampus compared to littermate controls. pCREB(Ser133) levels were unaffected in the cerebellum of rTg4510 mice. (B) Summary of the ratios of pCREB(Ser133) to total CREB in the cerebellum, cortex, and hippocampus (n = 5 for each genotype; p<0.01).
littermate controls (n = 5). Western blot analyses were conducted with tissue homogenates prepared from the cerebellum, cortex, and hippocampus of these two groups of mice (Figure 4.3). pCREB (Ser133) but not total CREB was significantly reduced in both the cortex and hippocampus of rTg4510 mice compared to control mice (Figure 4.3A; Table 4.1). The ratios of pCREB(Ser133) to CREB in the cortex or hippocampus of rTg4510 mice was less than 40% of control values, while no difference was found in the cerebellum (Figure 4.3B). These data suggest that cAMP/CREB signaling is impaired in rTg4510 brains prior to the onset of age-dependent cognitive deficits.

4.3 nmnat2 transcription could be regulated in a CREB-dependent manner

To examine whether reduced CREB activity can account for nmnat2 down-regulation in rTg4510 forebrains, an in silico analysis of the mouse nmnat2 promoter region was performed to search for possible CREB response elements (CRE) using Genomatix. This analysis identified two putative CREs close to the transcriptional start site of the mouse nmnat2 gene (Figure 4.4A). To test for potential pCREB binding to the putative CREs, chromatin immunoprecipitation experiments were conducted with a pCREB (S133) antibody, using both mouse whole-brain extract and extracts from 293T cells treated with either DMSO or forskolin (5 µM). Since previous reports have indicated a lack of NMNAT2 expression in 293T cells (Berger et al., 2005; Mayer et al.; Raffaelli et al., 2002; Zhang et al., 2003), a luciferase vector with full nmnat2 promoter (Pro-2Kb,
Figure 4.4A) was over-expressed in these cells to detect binding of pCREB to either CREs. ChIP analysis found direct binding of pCREB to the genomic regions containing either both nmnat2 CRE sites or only the CRE2 region in wild type brain (Fig. 4B). In addition, analysis in 293T cells also found pCREB binding to these regions and the amount of pCREB bound to the specific CRE-containing regions was increased upon forskolin treatment (Figure 4.4B).

Next, a Dual Luciferase assay tested the transcriptional functionality of the nmnat2 CRE sites. The nmnat2 promoter region that either included two kilobase (Kb) or 700 base pairs (bp) upstream and 200 bp downstream of the transcriptional start site (TSS) was cloned upstream of a luciferase expression cassette. The Pro-0.7Kb luciferase vector was then used as a template for site-directed mutagenesis of the specific CREs in various combinations (Figure 4.4A). To ensure a completely nonfunctional CRE, the core 5’-TGACGC-3’ sequence was mutated to 5’-TGGGTA-3’. A luciferase vector with three consecutive CREs derived from the achorionic gonadotropin promoter (AG CRE) was used as a positive control (Cruzalegui et al., 1992). Furthermore, to ensure that any enhanced luciferase induction upon forskolin treatment was due to pCREB binding to either CREs, the cells were transfected with either pcDNA-CREB or pcDNA-CREB^{S133A} (a dominant-negative pCREB mutant), in addition to the specific firefly and Renilla luciferase vectors (Figure 4.4C). Luciferase activity of cells transfected with vectors containing either AG CRE or nmnat2 promoter (Pro-2Kb or Pro-0.7Kb) was significantly increased upon forskolin treatment
Figure 4.4 *nmnat2* transcription is regulated by CREB. (A) Diagram of the mouse *nmnat2* promoter showing the sequence of the two predicted CREs close to the transcriptional start site (TSS). Schematic representation displaying the different inserts in firefly luciferase vector. (B) ChIP assay with pCREB antibody using mouse whole-brain lysate and 293T cells transfected with 2KB-Pro luciferase vector and treated with either DMSO or forskolin. pCREB binds to both CRE1+2 and CRE2 containing regions in wild type brain and 293T cells. Binding is enriched in 293T cells upon forskolin treatment. (C) Luciferase activity measured from HEK293 cells transfected with either pcDNA-CREB or pcDNA-CREBS<sup>S133A</sup> and either empty, AG CRE, *nmnat2*-promoter plasmid (Pro-2KB or Pro-0.7Kb) as well as CRE mutants CRE1, CRE1, CRE2 or mCRE1/2 after 12 hours of treatment with or without 5 µM forskolin (*P < 0.05, **P<0.01, ***P<0.001). Data are plotted as mean ± S.E.M (n =3 for each group).
(Figure 4.4C). The luciferase activity driven by the nmnat2-promoter (Pro-2Kb) increased almost five times from its basal level (4.7 ± 0.5 % of normalized Renilla luciferase activity) after forskolin treatment (20.4 ± 0.7 % of normalized Renilla luciferase activity; P = 0.001 between control and forskolin conditions). The luciferase activity driven by AG CRE was increased about eight fold from its basal level (6.16 ± 0.10 % of normalized Renilla luciferase activity) upon forskolin treatment (51.52 ± 8.53 % of normalized Renilla luciferase activity; P = 0.011 between control and forskolin conditions). Almost no increase in luciferase activity was detected with the vector containing one or two mutated CRE sites in either control medium or in forskolin containing medium. Furthermore, the induction of luciferase activity seen with AG-CRE, Pro-2Kb or Pro-0.7Kb upon forskolin treatment was absent in cells transfected with the dominant-negative pcDNA-CREBS133A, strongly suggesting the increase in luciferase activity was due to CREB phosphorylation. Since forskolin induces CREB phosphorylation indirectly via increasing the activity of adenylyl cyclase, this assay was repeated to measure the luciferase activity of all the constructs in the presence of a constitutively active form of CREB (CREBDIEDML). In cells transfected with CREBDIEDML, the level of luciferase activity is significantly high in cells transfected with either AG CRE, Pro-2Kb or Pro-0.7Kb (Fig. 4.4C). This indicates that the induction of luciferase activity is directly from the binding of pCREB to the nmnat2 promoter.
So far, the data indicate that endogenous nmnat2 is under the regulation of pCREB via both the functional CREs proximal to the transcription start site in its promoter. However, in P301L tauopathy model, CREB activity is significantly reduced, which could account for reduction in nmnat2 transcription. To test directly if pCREB binding is indeed reduced in rTg4510 cortex and hippocampus, where mutant Tau is overexpressed, additional ChIP assays were preformed to compare pCREB promoter occupancy of nmnat2 in tissues from diseased and wild type littermates (Fig. 4.5). In fact, pCREB occupancy was studied in the promoter region containing both CRE1 and CRE2 as well as at CRE2. For ChIP, both cortex and hippocampus tissue were used, where mutant Tau was overexpressed from 2 month old animals, and the cerebellum from the same animal served as an internal control. Compared to wild type tissue, pCREB occupancy was significantly reduced in rTg4510 cortex and hippocampus at both CRE1+CRE2 and CRE2 alone (Fig. 4.5). However, no such change is observed in the cerebellum, where pCREB promoter occupancy was similar between wild type and rTg4510. Quantification of promoter occupancy normalized to input was performed between rTg4510 and wild type cortex, hippocampus and cerebellum (n=3). This showed clear significant differences between promoter occupancy between wild type and rTg4510 cortex (p<0.05 for CRE1+CRE2; p<0.001 for CRE2) and hippocampus (p<0.001 for CRE1+CRE2 and p<0.05 for CRE2). Hence ChIP analyses revealed that indeed there is less pCREB binding to nmnat2 promoter region in tauopathy leading to a reduction in nmnat2 transcription by 2 months of age in these animals. Again, such a transcriptional
Figure 4.5 pCREB occupancy at nmnat2 promoter is reduced in rTg4510 cortex and hippocampus. ChIP assay was performed on cortex, hippocampus and cerebellum from 2 month old rTg4510 animals and wild type littermates. RNA polII was used as a positive control and rabbit IgG as a negative control. pCREB occupancy at nmnat2 promoter was studied in a region containing both CRE1 and CRE2 as well as one containing only CRE2. Quantification of pCREB promoter occupancy was performed using densitometry and normalized to input (n=3) (*p<0.05, **p<0.001).

dysregulation would precede any pathological features associated with neurodegeneration observed in tauopathies.

These data suggest that the nmnat2 promoter contains functional transcriptional elements responsive to pCREB (Seamon et al., 1981). Moreover, from the luciferase studies, both the CREs seem to be required for nmnat2 transcription and mutating either one abolishes transcription of this neuronal maintenance factor, suggesting transcriptional regulation in a cooperative manner. Together, this data suggest that cAMP/CREB signaling can regulate
nmnat2 transcription. Thus, the significant nmnat2 down-regulation in brain regions with tau\textsubscript{P301L} over-expression could be a direct consequence of reduced CREB activity.
In the previous chapter, the level of NMNAT2 was shown to be downregulated in an established mouse model of tauopathy. Furthermore, recent work has shown that overexpression of either human NMNAT1 or 2 is neuroprotective and rescues neurodegeneration in the hippocampus in this model of tauopathy (Ljunberg, C. and Ali, Y.O. et al., In Revision). The main goal in this chapter is to investigate the mechanism behind the neuroprotective capacity of NMNAT in a Drosophila model of tauopathy.

Previous work has shown that NMNAT displays chaperone function and NMNAT-mediated protection against neurodegeneration was partly through a proteasome-mediated pathway in a manner similar to heat-shock protein 70 (Hsp70) (Zhai et al., 2006; Zhai et al., 2008b). Several reports have implicated the therapeutic potential of chaperones in different models of tauopathy and other neurodegenerative conditions (Carmichael et al., 2000; Ostrerova et al., 1999; Sittler, 2001; Warrick et al., 1999). Specifically, in a cellular model of tauopathy, an inverse relationship between aggregated tau and the levels of HSP70/90 was observed, explaining a neuroprotective effect of overexpressing these heat shock proteins In the same study, overexpression of Hsp70 and 90 reduced hyperphosphorylated tau burden and promoted tau solubility by directly associating with tau aggregates (Dou et al., 2003). The mechanism of neuropro-
-tection availed by NMNAT in tauopathy has never been directly examined. The main hypothesis was that NMNAT can act like other chaperones on hyperphosphorylated tau to promote its clearance via ubiquitination.

Investigation of NMNAT’s role in tauopathy shows that NMNAT suppresses both the morphological vacuolization and the learning and memory decrements and locomotor deficits associated with pan-neuronal overexpression of human wild type and mutant tau in *Drosophila*. Importantly, comparable neuroprotection was observed from expressing either wild type or enzyme-inactive NMNAT (NMNAT\textsuperscript{WR}) in our tauopathy model. Extensive biochemical analyses revealed that NMNAT specifically binds human tau and disease-associated hyper-phosphorylated tau. Overexpression of either wild type or enzyme-inactive NMNAT reduced the levels of hyperphosphorylated tau oligomers and promoted clearance of hyperphosphorylated tau oligomers through ubiquitination, in a proteasome-mediated manner. Consequentially, tau induced age-dependent neurodegeneration was suppressed by NMNAT overexpression as indicated by reduced level of apoptosis and levels of activated caspase 3. Hence, the neuronal maintenance factor NMNAT can offer significant protection in tauopathy, and the molecular basis for NMNAT mediated protection is through promoting clearance of toxic hyperphosphorylated tau oligomeric species.
5.1 NMNAT expression rescues learning and memory and locomotor deficits induced by human tau overexpression

Tauopathies, such as Alzheimer's disease, are characterized by accumulation of abnormally phosphorylated and aggregated tau, leading to synaptic dysfunction underlying age-dependent severe dementia (Hutton et al., 2001; Lewis et al., 2000; Wittmann et al., 2001). It has been shown that overexpression of wild type tau in the adult Drosophila mushroom body neurons, centers of olfactory learning and memory, manifest in compromised associative olfactory learning and memory, significantly preceding the onset of visible neuronal loss and neurodegeneration (Mershin et al., 2004), suggesting that compromised behavioral plasticity may be the earliest detectable manifestations of taupathies.

To investigate whether neuronal maintenance factor NMNAT offers protection in Tauopathy, first learning and memory functions and locomotor deficits induced by tau expression were examined with or without NMNAT overexpression.

To model tauopathy in Drosophila CNS, either wild-type or Arg^{406}→Trp (R406W) mutant tau, an isoform associated with an early onset familial form of dementia, was over-expressed with a pan-neuronal elav-GAL4 driver (Wittmann et al., 2001). In addition, either wild-type or enzyme-inactive NMNAT (NMNAT^{WR}) was simultaneously over-expressed to assess its protective capacity in this neurodegenerative model. In NMNAT^{WR}, two of the key residues required for substrate binding are mutated, resulting in less than 1% enzymatic activity of the
wild type protein (Zhai et al., 2006). To control for tau expression dosage in different genotypes, GFP was over-expressed whenever NMNAT was not.

To measure learning and memory function, an aversive phototaxis suppression (APS) assay was established (Ali, 2011; Le Bourg and Buecher, 2002), where flies were forced to associate an aversive stimulus (bitter taste from quinine) with light and learn to avoid light and suppress phototaxis. Wild type flies are able to learn the task after ten training trials (Ali, 2011; Le Bourg and Buecher, 2002). Learning ability can be tested in 5 consecutive trials immediately after the training phase (PC0), while memory capacity can be tested in five trials 6 hours after the training phase (PC6). A successful avoidance of light in the test trial was considered as ‘pass’ and the passing rate for all trials was calculated and plotted as indexes for learning or memory abilities. The hypothesis here was that Tau flies will exhibit impaired behavioral plasticity and will have compromised learning and memory function. As shown in Figure 5.1A and Tables 5.1 and 5.2, overexpression of Tau or Tau^{R406W} in the CNS did not affect the learning (PC0) and memory (PC6) abilities of 2 day old flies, but resulted in significantly reduced PC0 and PC6 in 20-day old flies, suggesting that human Tau expression in Drosophila CNS causes learning and memory deficits in an age-dependent manner. Interestingly, loss of one copy of endogenous NMNAT (nmnat/+) significantly reduced both learning and memory abilities in 2 day old (2 DAE, days after eclosion) flies overexpressing Tau^{R406W}, suggesting that reduced endogenous NMNAT level caused an earlier onset of the impairment in learning
Figure 5.1 NMNAT rescues learning and memory deficits and locomotor impairments induced in tauopathy. (A) Learning and memory functions were studied in flies of specified genotypes using aversive phototaxis suppression assay, where flies were conditioned to associate light with the bitter taste of quinine. PC0=Learning Index; PC6= Memory Index. Overexpression of Tau or R406W causes age-dependent decrements in learning and memory functions. NMNAT or WR overexpression rescues these deficits. Error bars represent results in five consecutive trials for 20 individual flies of each age and genotype. (Statistical significance noted in Tables 1 and 2). (B) Locomotor performance was investigated using negative geotaxis assay. Overexpression of Tau or R406W cause significant deficit in locomotor activities which are rescued by NMNAT or WR. Each bar graph is an average of ten trials of hundred flies of each genotype and age. *p<0.05

and memory and exacerbated the deficit, initiated by mutant Tau overexpression.

Conversely, overexpressing NMNAT in CNS significantly improved Tau induced
learning and memory deficits in 20 day old (20 DAE) flies. Importantly, overexpression of NMNATWR has comparable neuroprotective activity as wild type NMNAT overexpression (Figure 5.1A, Table 5.1 and 5.2). These results suggest that Tau expression in the CNS induced age-dependent learning and memory impairment that can be suppressed by NMNAT overexpression. The neuronal level of NMNAT is an important determinant of the age of onset as well as the severity of the deficit, as reduced level of NMNAT lowered the age of onset of learning and memory deficits, while overexpressing NMNAT significantly improved learning and memory functions.

In addition to learning and memory functions, we next tested locomotor activity using negative geotaxis assay (Ali, 2011; Benzer, 1967; Greenspan et al., 1980). As shown in Figure 5B, overexpression of either Tau or TauR406W in the CNS caused an age-dependent decrease in locomotor activity as measured by climbing performance. However, overexpressing NMNAT or NMNATWR restored the locomotor performance close to wild type level. The results from behavioral analyses showed that, first, overexpressing Tau or TauR406W in the CNS caused an age-dependent decrease in learning and memory abilities and locomotor activity; second, the behavioral deficits were attenuated by overexpressing NMNAT; and third, the level of NMNAT is an important determinant for the age of onset and the severity of the behavioral deficits.
Table 5.1 Statistical significance of differences in PC0 between genotypes at 2- and 20-DAE age. Significance between mean PC0 from atleast 12 individual flies of each genotype per age was calculated using ANOVA test.

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Table 5.2  Statistical significance of differences in PC6 between genotypes at 2- and 20-DAE age. Significance between mean PC0 from atleast 12 individual flies of each genotype per age was calculated using ANOVA test.

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Day 20
5.2 NMNAT rescues age-dependent vacuolization induced by Tau expression

Previous work has shown that overexpression of Tau in Drosophila results in an age-dependent vacuolization and degeneration of cells in the cortex as well as in neuropil structures (Wittmann et al., 2001). Such vacuolization is more severe in flies overexpressing mutant Tau^{R406W}. To assess whether NMNAT can rescue this Tau-induced morphological change and neurodegeneration at the cellular level, the morphology of fly brain at 2- and 20-DAE was examined with immunofluorescence labeling for NMNAT, pTau^{Ser262} and DAPI (Figure 5.2). The level and distribution of Tau phosphorylation at serine 262 (pTau^{Ser262}) was examined because phosphorylation at this residue has been shown to be absolutely essential to mediate toxicity in established Drosophila models of Alzheimer's disease (Iijima et al., 2010). At 2 DAE, vacuolization was not observed in Tau overexpressing fly brain (Figure 5.2A-D), but was present in Tau^{R406W} overexpressing fly brain (Figure 5.2E-H, vacuole indicated by white arrowheads). At 20 DAE, multiple large vacuoles can be observed in both Tau and Tau^{R406W} fly brain indicating severe neurodegeneration (Figure 5.2I-P). Overexpressing either wild-type (Figure 5.2Q-X) or enzyme-inactive NMNAT significantly reduced the number and size of these age-dependent vacuolization in 20 DAE flies (Figure 5.3). Interestingly, NMNAT overexpression also altered the distribution of Tau^{pSer262}. In Tau or Tau^{R406W} expressing brains, Tau^{pSer262} was accumulated with age and distributed in clusters or filamentous pattern (Figure 5.2B, F, J and N, white arrowheads). However in NMNAT co-expressing brains,
Figure 5.2 NMNAT suppresses Tau-induced morphological vacuolization. Tau or R406W overexpression causes age dependent vacuolization (A-P) demarked by white boundary. Brains were stained for pTau$^{\text{Ser262}}$ to detect hyperphosphorylated Tau and also NMNAT levels (Red) and DAPI to mark cell bodies. Overexpression of NMNAT significantly reduced the size and occurrence of vacuoles in 20 Day old flies (Q-X). Scale Bar = 20µm. Black arrows: filamentous Tau; white arrows demark vacuole boundary.
the level of $\text{Tau}^{\text{pSer262}}$ was significantly reduced and no clusters were observed (Figure 5.2R,V). These results suggest that NMNAT overexpression can suppress the morphological phenotypes and reduce Tau-induced neurodegeneration at the cellular level.

Figure 5.3 NMNAT reduces the total vacuole volume per brain induced by hTau overexpression. Over expression of both wild type and enzyme-deficient NMNAT reduce total vacuole volume per brain. Vacuole volume was quantified by using FluoView10-ASW (Olympus) software, assuming each vacuole to be spherical in form. Numbers at the bottom of the bars indicate the number of brains quantified per age per genotype. *$p<0.001$, **$p<0.05$
5.3 NMNAT reduces the level of Tau hyperphosphorylation

The significant rescue of Tau-induced behavioral and morphological impairments by either wild type or enzyme-inactive NMNAT suggested that the mode of protection availed by NMNAT might possibly have an enzyme-independent mechanism. Studies using tauopathy models have suggested the main cause of neurodegeneration being tau hyperphosphorylation and oligomerization that interfere with proper neuronal functions and lead to cell death (Blard et al., 2006; Dickey et al., 2007; Iijima et al., 2010; Steinhilb et al., 2007b; Wittmann et al., 2001). In order to reveal the mechanism underlying the protective effects of NMNAT, the impact of NMNAT expression on the levels of hyperphosphorylated tau was thoroughly investigated.

To determine the conformation and phosphorylation state of Tau protein, specific antibodies were used to detect tau abnormalities in disease state: AT8 and AT180 antibodies to preferentially recognize tau phosphorylation characteristic of Alzheimer’s disease state (Jicha et al., 1997a; Jicha et al., 1997b; Wittmann et al., 2001), Tau^{pSer262} antibody to probe for serine 262 specific phosphorylation of tau, as the phosphorylation at this residue has been shown to be essential for neurotoxicity associated with a different model of Alzheimer’s disease (Iijima et al., 2010), and human Tau antibody to probe the total level of Tau protein expressed. The specificity of these antibodies was indicated by lack...
Figure 5.4 NMNAT reduces the levels of hyperphosphorylated, disease-associated Tau oligomers. (A-B) Brain lysates of 2 or 20-DAE wild type flies (lane 1-2) or flies overexpressing GFP (lane 3-4), hTau (WT (A) or R406W (B)) with GFP (lane 5-6) or NMNAT (lane 7-8) or NMNAT<sup>WR</sup> (lane 9-10), as well as in the <i>nmnat</i>/+ background (lane 11-12) were probed with antibodies specific for disease-associated phospho-Tau epitopes AT8, AT180 and Tau<sup>Ser262</sup> as well as total hTau and Actin for loading control. (C-D) Quantification of phosphorylated-Tau species normalized to Actin and Total hTau from samples in A in 2-DAE (C) and 20-DAE (D) flies or B in 2-DAE (E) or 20-DAE (F) flies. *p<0.05
of immunoreactivity in the control flies that were not expressing human Tau (Figure 5.4A, B, lanes 1-4). In Tau or Tau^R406W expressing flies, a significant level of phosphorylated Tau was detected in the CNS by all three phospho-specific antibodies (Figure 5.4A, B, lanes 5-6). When NMNAT or NMNAT^WR was co-overexpressed, a significant decrease in phospho-Tau was detected by AT8, AT180 and pSer262 antibodies in 2 DAE flies (Figure 5.4A, B lane 7 and 9, quantifications in 5.4C and 5.4E). The level of phospho-Tau was higher in TauR406W expressing flies than that in Tau flies, consistent with a more aggressive phenotype produced by Tau^R406W overexpression (Wittmann et al., 2001). Phospho-Tau accumulates with age as the levels were substantially elevated in 20 DAE Tau or TauR406W expressing flies, and overexpressing NMNAT provided significant reduction in the level of phospho-Tau (Figure 5.4A, B, lanes 8 and quantifications in 5.4D and 5.4F). More interestingly, removing one copy of endogenous NMNAT significantly increased the level of phospho-Tau in Tau^R406W expressing flies, (Figure 5.4A, B, lanes 11 and 12, and quantifications in 5.4C-5.4F), consistent with the observation that removing one copy of endogenous NMNAT exacerbated the learning and memory defect caused by Tau^R406W overexpression (Figure 5.1). These results suggest that the level of phospho-Tau is correlated with the severity of the neurodegenerative phenotypes and that NMNAT-mediated suppression of Tau-induced behavioral and morphological deficits is the result of reduced level of hyperphosphorylated Tau, hence mitigating toxicity caused by such modifications.
5.4 NMNAT interacts with Tau oligomers

Next, the mechanism underlying NMNAT mediated reduction of hyperphosphorylated Tau was examined. Previous work on the biochemical properties of NMNAT has uncovered an enzyme-independent chaperone function that contributes to its neuroprotective effects against neurodegeneration in models of spinocerebellar ataxia 1 (SCA1) (Zhai et al., 2008b). NMNAT was shown to be recruited with chaperone heat shock protein 70 (Hsp70) into hATX-82Q aggregates and reduce aggregation load partly through a proteasome-mediated pathway (Zhai et al., 2008b). I hypothesize that the chaperone function of NMNAT may contribute to the clearance of toxic phosphor-Tau oligomeric species. To test this hypothesis, first it was tested if NMNAT interacts directly with overexpressed human Tau proteins by immunoprecipitation analysis using brain lysates from Tau or Tau R406W expressing flies. As shown in Figure 5.5A, endogenous NMNAT specifically interacted with Tau and phosphor-Tau proteins in Tau or Tau R406W expressing fly brains. Next, to uncover the mechanism of NMNAT mediated Tau clearance, the state of Tau ubiquitination was investigated in detail as several studies have reported the involvement of the ubiquitin-proteasome in Tau degradation (Babu et al., 2005; Blard et al., 2006; Cardozo and Michaud, 2002; David et al., 2002; Goldbaum et al., 2003; Hatakeyama et al., 2004; Petrucelli and Dawson, 2004; Petrucelli et al., 2004; Shimura et al., 2004; Zhang et al., 2005). Moreover, Tau extracted from post-mortem AD brains but not from control brains was shown to be heavily ubiquitylated by the E3 ubiquitin ligase CHIP in vitro (Khatoon et al., 1992). Hence, it is possible that
NMNAT overexpression can promote ubiquitination of Tau oligomers. To test this, either wild type or enzyme-inactive NMNAT was overexpressed in Tau\(^{R406W}\) expressing fly brains from which total human Tau was immunoprecipitated. As shown in Figure 5.5B, western analysis with a ubiquitin-specific antibody showed that overexpression of NMNAT or NMNAT\(^{WR}\) promoted the clearance of lower molecular weight (250 kDa and less) ubiquitinated Tau oligomers (Figure 5.5B).

**Figure 5.5 Endogenous NMNAT interacts with hTau oligomers to promote proteasome-mediated clearance of ubiquitinated Tau.** (A) Immunoprecipitation of whole brain lysates with NMNAT antibody were probed with total hTau or disease-associated phospho-Tau specific antibody AT8 and AT180. Lack of Tau bands in the negative Wild type and GFP overexpressing brains reveal that the NMNAT-Tau interaction is specific to overexpressed hTau. (B) Total hTau was immunoprecipitated from brains of 2-DAE flies overexpressing wild type hTau, hTau\(^{R406W}\), hTau\(^{R406W}\) + NMNAT, hTau\(^{R406W}\) + NMNAT\(^{WR}\) and probed for ubiquitin. Stacking gel shows HMW Tau oligomers and Lower Gel shows LMW oligomers (note the arrowheads) that can be monoubiquitinated. Note in NMNAT overexpressing flies, the band immunoprecipitated at 75kDa probably corresponds to ubiquitinated hyperphosphorylated Tau that can be directly cleared by the proteasome.
arrowheads), while increasing the levels of ubiquitinated Tau monomer (Figure 5.5B, ~75 kDa bands indicated by an arrow).

These results indicate that NMNAT overexpression reduces the mutant human Tau-induced toxicity by promoting the clearance of the lower molecular weight ubiquitinated Tau oligomeric species via the ubiquitin-proteasome pathway. In addition, higher molecular weight (above 250 kDa) ubiquitinated Tau species were observed as a smear in the stacking gel (Figure 5.5B), which were hardly detectable in Tau or TauR406W expressing flies (Figure 5.5B, lanes 3 and 6), but significantly increased with NMNAT overexpression (Figure 5.5B, lanes 9 and 12), suggesting that NMNAT may be able to promote the formation of higher molecular weight Tau oligomers that are far less toxic than low molecular weight Tau oligomers. These results suggest that NMNAT directly interact with Tau proteins and promote the clearance and breakdown of toxic oligomeric Tau species through the ubiquitin-proteasome pathway and facilitate the formation of less toxic high molecular weight Tau aggregates, therefore reduce the neuronal load of highly toxic oligomeric Tau species.

5.5 NMNAT protects against Tau-induced Apoptosis

Neuronal overexpression of hTau in Drosophila is shown to induce apoptosis resulting in age-dependent vacuolization (Wittmann et al., 2001). So far overexpression of NMNAT was shown to suppress human Tau induced
Figure 5.6 NMNAT-mediated protection of morphological defects in Tauopathy is partly through reduction in apoptosis. (A-B) Levels of apoptosis were measured in 2- and 20-DAE wild type flies (yw, lane 1-2) and flies overexpressing GFP (lane 3-4); wild type hTau (A) or hTau^{R406W} (B) with GFP (lane 5-6) or NMNAT (lane 7-8) or NMNAT^{WR} (lane 9-10) or in the nmnat/+ background (lane 11-12) by probing for cleaved caspase 3, total caspase 3 and actin as a loading control. (C-D) Quantification of cleaved caspase-3 levels in A (C) or B (D) flies, normalized to total caspase3 and actin.
neurodegeneration, and reduce hyperphosphorylated tau levels by promoting ubiquitination and clearance. Next the overall level of apoptosis was measured in this model to test whether NMNAT expression affects the level of apoptosis caspase 3 (Figure 5.6A-B, lanes 5 and 6), induced by Tau overexpression. As shown in Figure 5.6, overexpression of either Tau or Tau\textsuperscript{R406W} cause significant increase in the levels of cleaved consistent with previous observation (Fulga et al., 2007; Gamblin et al., 2003). However, overexpressing NMNAT or NMNAT\textsuperscript{WR} significantly reduced caspase3 activation via cleavage in both D2 and D20 flies (Figure 5.6A-B, lanes 7-10, quantifications in 5.6C-D). In contrast, loss of an endogenous copy of NMNAT (\textit{nmnat/+}) caused a significant increase in cleaved caspase 3 levels (Figure 5.6A-B, lanes 11-12, quantifications in 5.6C-D). These results suggest that human Tau overexpression induces caspase activation and apoptosis, and higher levels of NMNAT expression significantly reduced the level of caspase 3 activation and the resulted apoptosis, while reduced level of NMNAT expression exacerbated apoptosis induced by Tau expression.
Chapter 6. NMNAT Interacts With Endogenous Client Proteins

Under normal conditions, NMNAT is present at high levels in cells, localized to the cell body as well as the synapse in a neuron (Zhai et al., 2006). In particular, localization studies in third instar larvae show NMNAT to be abundantly expressed in neuronal nuclei in the brain and ventral nerve cord (unpublished data) and in muscle cell nuclei (Figure 6.1 A-C) (Zhai et al., 2006). In addition, NMNAT is also present at the neuromuscular junction where it partially co-localizes with the active zone marker nc82 (Figure 6.1 A-C) (Zhai et al., 2006). In the pupal optic lobe, NMNAT levels are detected in the cell bodies of photoreceptors and numerous optic lobe neurons (Figure 6.1 D-F). Furthermore, in adult optic lobe, NMNAT is expressed in the cell bodies of photoreceptors as well as in the neuropil structures of the lamina and at the photoreceptor terminals (Figure 6.1 G-I). The overall analysis of NMNAT localization reveals that NMNAT is enriched in the nervous system, both in the neuronal nuclei and the nerve terminals, present at high levels in wild type animals (Zhai et al., 2006).

The data so far suggests that NMNAT is localized in neurons at the synapse and also the cell body at relatively high levels. Furthermore, NMNAT is also upregulated at both the transcript and protein levels post acute stress (Ali et al, 2011) and also acts as a chaperone to prevent protein misfolding as seen in its neuroprotection strategy in tauopathies (Chapter 5). Comparison with other chaperones categorizes NMNAT in a class of chaperones that are both constitut-
Figure 6.1 Characterization of NMNAT localization in Drosophila nervous system. (A–C) Third instar larval neuromuscular junction (NMJ). (D–F) Optic lobe (P+60%) immunolabeled for Synaptotagmin (syt) to mark synaptic vesicles (blue), with nc82 to mark active zones (green), and for NMNAT (red). la, lamina; lc, lamina cortex; mc, medulla cortex; me, medulla. (G–I) MARCM analysis of adult lamina. GFP marks the mutant patches. NMNAT labeling appears as a punctate pattern decorating nc82 labeling. (J–L) Adult brain NMNAT staining. AL, antenna lobe; MB, mushroom body. Scale bars indicate 5 µm. (Adapted from Zhai et al, 2006, PLoS Biology.)
-ively expressed and induced upon stress, such as Hsp90 (Ali et al, 2010). Hsp90 is abundantly present in most cellular compartments including cytosol, endoplasmic reticulum (ER), mitochondria (and chloroplast in plants), under unstressed conditions (Taipale et al., 2010). Hsp90 is indispensible for cell survival, playing an important role in the folding of at least 200 specific proteins in essential signaling pathways, and in the refolding of denatured proteins after stress (Akner et al., 1992; Biggiogera et al., 1996; Langer et al., 2003; Pratt and Toft, 1997). Since NMNAT also behaves like Hsp90 and is localized in distinct cellular compartments, it supports possible interactions of NMNAT with distinct protein partners, that might also help elucidate the mechanism of degeneration observed in the loss-of-function NMNAT mutants.

6.1 NMNAT interacts with Bruchpilot to promote active zone maintenance at the synapse

In order to specifically explore the role of NMNAT at the active zone, whole adult brain lysates were used to immunoprecipitate NMNAT to study possible interactions with active zone and synaptic proteins. Out of all the proteins investigated to possibly interact with NMNAT, Brp was the only active zone protein shown to immunoprecipitate with NMNAT (Fig. 6.2A-B). This explained the colocalization between NMNAT and BRP observed in L3 larval neuromuscular junctions (Fig. 6.1 A-C). The interaction was specific as NMNAT
did not immunoprecipitate with other active zone proteins such as DLAR (Fig. 6.2D) or synaptic proteins such as synaptotagmin (Fig. 6.2 C).

Recent work identified BRP as an integral component of the T bar (Fouquet et al., 2009). The N-terminal of BRP shares significant homology to CAST protein, while the C-terminal shares similarity to large cytoskeletal proteins (Wagh et al., 2006). Moreover, BRP was shown to be essential for structural and functional integrity of the active zone (Wagh et al., 2006). Loss of BRP results in a complete loss of T-bars and a diminished density of Ca^{2+} channel clusters at active zones (Kittel et al., 2006a; Kittel et al., 2006b). Such an interaction with Brp could explain the possible cause of synaptic degeneration observed in nmnat mutant photoreceptors.

**Figure 6.2 NMNAT interacts with the active zone protein, Brp, in vivo.** Immunoprecipitation of wild type Drosophila brain lysate with antibodies specific for NMNAT (A, C-D) and BRP (B) and probed with BRP (A), NMNAT (B), synaptotagmin (C), DLAR (D). (E) Western blotting of NMNAT and Brp levels in control and BRP- / NMNAT-RNAi fly brain lysates. (F) Quantification of NMNAT and Brp levels normalized to actin.
Moreover, this interaction is further confirmed in adult brains where NMNAT-RNAi is driven with a pan-neuronal driver (Fig. 6.2E-F). Here, when NMNAT is knocked down, Brp levels are also significantly reduced. However, when Brp is knocked down using an RNAi line driven by the same pan-neuronal driver, NMNAT levels are not affected. This raises the possibility that NMNAT might be required at the active zone to maintain stable levels of Brp. Further work is currently under way to uncover the possible consequences of this interaction, with respect to NMNAT’s role in maintaining the stability of the active zone.

6.2 NMNAT binds microtubules to promote stability

A similar protein-protein interaction assay that relied on immunoprecipitating NMNAT from adult wild type Drosophila brains, showed that NMNAT interacts with tubulin (Figure 6.3). Specifically, this interaction was seen to be strong when the NMNAT immunoprecipitate was probed for alpha-tubulin (Fig 6.3 A) and acetylated tubulin (Fig. 6.3C). The reverse immunoprecipitation reaction using alpha tubulin antibody confirmed this interaction as it pulled down NMNAT (Fig. 6F).

This interaction of NMNAT with tubulin raised the possibility that it might possibly interact with microtubules directly. Previous studies on the neuroprotective effects of NMNAT on axon degeneration showed that post injury, NMNAT-infected dorsal root ganglion explants were more resistant to
degeneration and had more acetylated tubulin, which might result from more stable microtubules. As shown in Fig. 6.3G, NMNAT is capable of binding to preformed microtubules, with the same efficacy as MAP2 (positive control), which is seen to pull down with the microtubule pellet.

**Figure 6.3** NMNAT interacts with microtubules *in vivo*. Immunoprecipitation of wild type Drosophila brain lysate with antibodies specific for NMNAT (A-E) and alpha-tubulin (F) and probed with alpha-tubulin (A), gamma-tubulin (B), acetylated tubulin (C), NMNAT (D), DLAR (E) and NMNAT (F). (G) NMNAT binds to preformed microtubules *in vitro*. Both NMNAT and Map2 pulls down with microtubule pellet, unlike the negative controls, Tropomyosin and BSA.

Uncovering some of the endogenous client protein of NMNAT helps us understand why NMNAT is so essential for neuronal maintenance. For example, understanding the interaction of NMNAT with BRP, which forms the active zone
backbone in *Drosophila*, provides insight into why loss of NMNAT causes synaptic degeneration, where increased activity calls for increased synaptic maintenance. At the same time, observations of increased axonal protection post injury, from NMNAT overexpression in DRG explants, could be explained by increased microtubule stability via direct binding of NMNAT in axons. Future work on how NMNAT's interaction with either BRP or microtubules promotes neuronal maintenance is underway.
Chapter 7. Discussion

7.1 Insights into the role of NMNAT as a stress protein

My studies in this dissertation show that Drosophila NMNAT is a stress response protein essential for thermotolerance and mitigation of shortened lifespan in flies subjected to Paraquat-induced oxidative stress (Ali et al., 2011). NMNAT is up-regulated in vivo under various stress conditions including heat stress, hypoxia, and oxidative stress (Ali et al., 2011). The stress transcription factor HSF is the central regulator of nmnat transcription upon stress (Ali et al., 2011). The transcriptional response of NMNAT under stress is consistent with its chaperone function (Zhai et al., 2008b), further suggesting that the housekeeping enzyme NMNAT can respond transcriptionally to stress conditions by means of increased protein levels and mitigation of cellular proteotoxicity. My findings expand the cellular stress network beyond the realm of heat shock proteins to include a metabolic enzyme. Although a few other enzymes have been found to be up-regulated upon stress (Cramer et al., 1995; Lee, 1992), the work in Chapter 3 provides a first example of a metabolic enzyme as an integral part of the stress response inasmuch as partial loss of nmnat significantly reduces stress tolerance.

The upregulation of NMNAT under stress shares significant similarity to heat shock protein Hsp70. However, important differences were observed betw-
een NMNAT and Hsp70. First, under normal conditions, in contrast to Hsp70, which is not expressed (Kabani and Martineau, 2008), NMNAT is expressed at moderate levels because of its function as a housekeeping enzyme. Second, although both NMNAT and Hsp70 are directly regulated by transcription factor HSF, a significant difference in the relative protein levels between Hsp70 and NMNAT was observed in HSF-overexpressing flies. For example, NMNAT was expressed at a high level in HSF OE flies prior to heat shock and was induced further upon heat shock, whereas Hsp70 was expressed at high levels prior to heat shock but was maintained at the same expression level upon heat shock (Fig. 1.2A). This is likely due to the negative feedback mechanism that regulates Hsp70 expression (Abravaya, 1992; Morimoto et al., 1992), where excess Hsp70 protein binds to HSF, reducing the DNA binding capacity of HSF, thereby attenuating transcription of Hsp70 and other HSPs (Abravaya, 1992; Feder et al., 1992; Morimoto et al., 1992). Therefore in HSF OE flies, the level of Hsp70 prior heat shock is already at a high enough level to inhibit further induction upon heat shock. In contrast to Hsp70, NMNAT is a housekeeping enzyme and is constitutively expressed at a moderate level under normal conditions (Zhai et al., 2008b). The up-regulation of NMNAT under stress in HSF-overexpressing flies was persistent without decline (Figure 3.5), indicating that NMNAT may not be under the same negative feedback regulation as Hsp70. These differences in transcriptional regulation between NMNAT and heat shock proteins suggest that NMNAT, as a housekeeping metabolic enzyme, may represent a different class of stress response proteins. Inasmuch as housekeeping enzymes are readily
available under normal conditions, they can be the first responders to a stress condition and thereby reduce the resultant proteotoxicity.

Several microarray and expression profiling studies in different organisms have indicated stress-related change in the expression of NMNAT homologues. For example, treating parental salt-sensitive rats with mild hypoxia (12% oxygen) revealed a 1.96-fold up-regulation of NMNAT1 transcripts ($p<0.0005$) (Bruder et al., 2007), and treating the quadriceps of mice in hypoxic conditions for 2 weeks induced a 2.23-fold up-regulation of NMNAT1 transcript levels ($p=0.0079$) (Laifenfeld et al., 2010). In Saccharomyces cerevisiae, a 45-min exposure to anoxic conditions induced a robust up-regulation of NMNAT transcription (fold change, 1.8; $p=0.04$) (Chan and Roth, 2008). Such conservation in NMNAT regulation upon stress is also evident in humans, where up-regulation of NMNAT may be an adaptive response to cope with stress from natural environment as seen in a study in Andeans living in high altitudes with chronic hypoxia who have higher levels of NMNAT1 compared with control subjects who live in lower altitudes (Appenzeller et al., 2006). Interestingly, the protein levels appeared to go down within an hour when the human subjects were brought down to sea level, suggesting that the elevated NMNAT1 levels at high altitude were a result of transcriptional control upon hypoxia (Appenzeller et al., 2006). The evolutionarily conserved transcriptional regulation of NMNAT expression further suggests the essential role of NMNAT in stress response.
My genetic studies indicated that stress transcription factor HSF is required for NMNAT up-regulation under heat shock and HIF1-α is required under hypoxia (Figure 3.5). Genomic analysis of the promoter region of nmnmt gene further revealed consensus binding sites for HSF and HIF1-α. However, my subsequent functional analyses indicated that the HIF1-α-binding element is not functional and that the up-regulation of NMNAT under hypoxia is indirectly mediated by HSF.

It is known that HIF1-α mediates the stress transcription during hypoxia by dimerizing with HIF1-α and binding to the consensus binding site A/GCGTG present in the HREs of many oxygen-regulated genes (Camenisch et al., 2001). It has been suggested that the HRE core sequence, (A/G)CGTG, is necessary but not sufficient for gene activation because functional HREs require flanking sequences with important DNA binding elements for additional transcription factors essential for transcription initiation. For example, HIF1-α cooperates with the ATF-1/CREB-1 factor in lactate dehydrogenase A gene transcription (Ebert and Bunn, 1998), with AP-1 binding factors in the VEGF gene transcription (Damert et al., 1997) and with the orphan receptor hepatic nuclear factor 4 in the erythropoietin HRE (Galson et al., 1995). In all these cases, the interaction of HIF1-α and other transcription factors is mediated by binding of HIF1 complex with p300 (Wenger, 2002). In addition, multimerization of the core consensus sequence is required to form a functional HRE in some genes. For example, in glucose transporter gene and several glycolytic enzymes more than two adjacent
core sequences form functional HREs (Wenger, 2000). In my promoter sequence analysis, I identified only a single consensus putative HRE site without flanking p300-binding sites or other possible transcription factor-binding sites in the close vicinity in the *nmnat* promoter. Therefore, it is likely that a lack of additional transcription factor-binding sites or a lack of multimerization of the core sequence renders this putative HRE nonfunctional.

Previous work has shown that loss of *Drosophila* NMNAT causes severe neuronal and synaptic degeneration without affecting neural development (Zhai et al., 2006), and overexpression of *Drosophila* NMNAT protects neurons from excessive activity-induced degeneration or proteotoxic neuronal degeneration (Zhai et al., 2006; Zhai et al., 2008b). Furthermore, my biochemical analyses uncovered a novel chaperone function independent of NAD enzymatic activity (Zhai et al., 2008b). In addition, structural analysis of human NMNAT1 and NMNAT3 and chaperone proteins in the entire Protein Data Bank revealed that the structures of these two proteins share significant similarity with chaperone UspA and Hsp100 (Zhai et al., 2009). Most of the structural similarities between NMNAT1 and UspA are at the helical core folds of NMNAT overlapping with that of UspA (Zhai et al., 2009). This additional chaperone function was consistent with the protective effects of NMNAT homologues in different neurodegenerative conditions, because chaperones such as Hsp70, Hsp40, and Hsp16.2 have also been shown to be protective in several neurodegenerative disease models including Alzheimer's disease (Fonte et al., 2008; Magrane et al., 2004),
amyotrophic lateral sclerosis (Gifondorwa et al., 2007), Parkinson’s disease (Auluck and Bonini, 2002; Auluck et al., 2002), Huntington’s disease, and other polyglutamine expansion disorders (Chan et al., 2000; Cummings et al., 1998; Cummings et al., 2001). Studies indicated in chapter 3 provide transcriptional mechanisms for the regulation of NMNAT expression and further support the role of NMNAT in stress response and neuroprotection. Taken together, these studies revealed that NMNAT proteins not only possess structural elements resembling other known chaperone proteins but also share a network of transcriptional regulatory machinery with heat shock proteins. The implication of such a stress response regulated by stress transcription factors is a cellular protective mechanism that can be harnessed to initiate novel therapies for proteostress-induced neurodegeneration.

The stress responsive property of NMNAT provides a functional link between cellular stress responses (e.g. heat shock, hypoxic, or oxidative stress) that includes synthesis and activation of molecular chaperones and key metabolic enzymes and protection of the proteome in neurons under normal (or healthy) versus neurodegenerative conditions. The HSF/HIF1 transcriptional pathway that I identified in NMNAT regulation revealed a network of the intricate and complex signaling and transcriptional circuits involved in the regulation of stress-responsive genes encoding neuroprotective proteins. Additional research toward unveiling this complex stress response network will prove both interesting and promising for the discovery of new therapeutic strategies for neuroprotection.
7.2 Role of NMNAT in Tauopathies

NMNAT has been shown to be an essential neuronal maintenance factor and its protective capacity is strictly maintained by the levels at which it is present. In fact, over-expression studies of NMNAT have shown this protein to provide neuroprotection against several degenerative conditions in Drosophila (Zhai et al., 2006; Zhai et al., 2008b). In mammals, over-expressing NMNAT isoforms have delayed Wallerian degeneration (Araki et al., 2004; Press and Milbrandt, 2008; Sasaki and Milbrandt; Sasaki et al., 2009; Wang et al., 2005; Yahata et al., 2009). Furthermore, NMNAT has been shown to act as a chaperone, a function independent of its housekeeping NAD-synthase function.

The role of NMNAT has not been studied extensively in context to neurodegeneration. Although NMNAT has been shown to protect against polyglutamine expansion induced neurodegeneration in a Drosophila model of SCA1 partly through a ubiquitin-proteosome mediated pathway, the chaperone function of this protein has not been exploited in tauopathies. The rationale behind choosing to study NMNAT’s neuroprotective role in this particular model was the fact that aggregation of tau into neurofibrillary lesions is a neuropathologic hallmark of many neurodegenerative diseases. Moreover, work from collaborators has shown AAV-mediated overexpression of both NMNAT1 and 2 to be neuroprotective in a murine model of tauopathy (unpublished). My main goal was to first study changes in endogenous NMNAT from hTau
overexpression in the rTg4510 murine tauopathy model; and second, use *Drosophila* to overexpress hTau and test the protective capacity of both wild type and enzyme-dead NMNAT.

### 7.2.1 Why is NMNAT2 downregulated in Tauopathy?

In the mouse model of tauopathy, I found that *nmannat2* mRNA was significantly reduced in brain areas overexpressing human Tau^{P301L} in one-month-old rTg4510 mice, an FTDP-17 tauopathy animal model. This was the time point when hTau^{P301L} expression was highest; in fact, after this decline in mRNA levels, NMNAT2 expression remained low throughout life. In an attempt to understand the mechanism behind this sharp reduction, transcriptional analyses demonstrated CREB-dependent regulation of *nmannat2* transcription through two CREB binding sites in the *nmannat2* promoter using a combination of ChIP and luciferase activity assays. The early reduction of pCREB (Ser133) levels in hTau forebrain suggests defects in CREB-mediated transcription and signaling as an early event in tauopathy. In fact, when rAAV over-expressed NMNAT2 or its homolog NMNAT1 in the hippocampus of rTg4510 mice, the extent of neurodegeneration was significantly reduced. Taken together, my work in chapter 4 demonstrates down-regulation of NMNAT2 and CREB activity in the CNS of a tauopathy animal model. Notably, these reductions precede the onset of neurodegeneration.
CREB is regulated via activity-driven signaling cascades and has been called “the memory gene” for several species (Alberini, 2009; Benito and Barco, 2010; Lee and Silva, 2009; Lonze and Ginty, 2002). Reduction in cCREB activity has been linked to deficits in both synaptic plasticity and long-term memory, whereas overexpression of CREB results in memory enhancements (Lee and Silva, 2009). Moreover, CREB has been shown to be vital for cell survival (Bito and Takemoto-Kimura, 2003; Lonze and Ginty, 2002). Previous work has linked deficiency in CREB activity to neurodegeneration whereby removing the CREB1 gene in adult mouse forebrain or chronic inhibition of CREB function led to extensive neurodegeneration in the hippocampus (Mantamadiotis et al., 2002; Valor et al., 2010). CREB affects transcription of its targets via binding to CREB Response Elements (CREs) present in the target’s promoter region (Impey et al., 2004). However, transcriptional regulation via CREB can only occur upon phosphorylation at Ser133 (Mayr and Montminy, 2001). Interestingly, pCREB immunoreactivity was significantly decreased in human Alzheimer’s disease brains (Yamamoto-Sasaki et al., 1999). As a suggestive mechanism, CREB signaling is affected in Alzheimer’s brains through deleterious effects of Aβ on hippocampal function (Espana et al., 2010; Gong et al., 2006; Smith et al., 2009; Vitolo et al., 2002). Supporting my work, pCREB levels were also shown to be reduced in 3xTg mice at six months of age (Caccamo et al., 2010), consistent with my findings (Fig. 4.3). I found that pCREB(Ser133), but not total CREB levels, are reduced in rTg4510 mice prior to the onset of neurodegeneration. No difference was found in the levels of p300, cREL or NFATc (data not shown),
transcription factors that have been implicated in different neurodegeneration models (Kogel et al., 2004; Martin-Loeches et al., 2001; Rouaux et al., 2003; Wu et al., 2010).

How might tauP301L overexpression reduce pCREB levels? Neuronal CREB activity is known to be increased by NMDAR (N-methyl-Daspartate receptors)-mediated calcium influx through several signaling pathways (Lonze and Ginty, 2002). A recent study by Hoover et al. (2010) found fewer NMDARs on dendritic spines of the same tauopathy model I used in my studies. They linked this reduction in NMDAR to the mislocalization of tau protein from axons to dendritic spines (Fig. 7.1). NMDAR-dependent long-term potentiation was impaired in the Schaffer collateral pathway of rTg4510 mice at 4.5 months of age. Aβ oligomers have also been linked to causing tau mislocalization (Ittner et al., 2010; Zempel et al., 2010). However, these studies fail to determine whether reduced NMDAR function in rTg4510 mice directly accounts for lower levels of pCREB. If so, therapeutically it would be interesting to see if augmentation of NMDAR signaling can reverse the NMDARs have been a popular target for enhanced cognition (reviewed in (Lee and Silva, 2009). Various transgenic and pharmacological approaches that augment NMDAR signaling have been found to increase learning and memory in mice, for example, NR2B overexpression and application of NMDAR partial agonist, D-cycloserine (Flood et al., 1992; Thompson et al., 1992).
In this study, reduced CREB activity in rTg4510 mice was found to be one of the major causes for the downregulation of nmnat2 transcription. I have identified two functional CREs upstream to the transcriptional start site of the mouse nmnat2 gene. Extensive promoter analysis demonstrated direct binding of pCREB to these CREs in mouse brains and mutating either one of the CREs was sufficient to abolish nmnat2 transcription. In addition to the reported regulation of NMNAT2 stability through proteosome pathway (Gilley and Coleman, 2010), my results provide additional insight into the multi-faceted regulation of this neuronal maintenance factor at the transcriptional level. From my analyses, nmnat2 is likely to be a CREB target necessary for maintaining neuronal health. In fact, small changes in CREB activity result in a rapid decline of nmnat2 levels, consistent with the labile nature of this protein. Multiple signaling pathways, such as the AKT pathway have been implicated in the regulation of pCREB activity and would offer tight regulation of NMNAT2 levels (Lonze and Ginty, 2002). My results suggest that tauP301L overexpression impairs CREB-mediated transcription and leads to a decrease in nmnat2 expression prior to the onset of neurodegeneration. The down-regulation of CREB activity in rTg4510 mice and the subsequent reduction of NMNAT2 could partially account for the cognitive deficits and neurodegeneration induced by tauP301L over-expression.
7.2.2 The mechanism of NMNAT-mediated neuroprotection in protein foldopathies

In the P301L Tau-Transgenic mouse, my collaborators have shown significant reduction in neurodegeneration associated with Tau-overexpression from AAV-mediated overexpression of NMNAT-1 and 2. To further understand the mechanism of NMNAT-mediated neuroprotection in tauopathy, I used Drosophila as a model to overexpress either wild type or mutant (R406W) human Tau with a pan-neuronal driver. Drosophila models of neuronal tauopathy exhibit key features of the human disorders including adult onset, age-dependent increase in neurodegeneration, accumulation of abnormal tau and compromised lifespan (Wittmann et al., 2001). Interestingly, neuronal tauopathy in Drosophila exhibits all these pathological hallmarks of the disease without forming neurofibrillary tangles, showing that the toxic species compromising behavior and lifespan seem to be the soluble hyperphosphorylated Tau oligomers (Wittmann et al., 2001).

Neuronal maintenance factor NMNAT has been shown to protect against poly-glutamine expansion induced neurodegeneration in a Drosophila model of SCA1 partly through a ubiquitin-proteosome mediated pathway (Zhai et al., 2006; Zhai et al., 2008b). The chaperone function of NMNAT predicted a possibility of a role of NMNAT in regulating protein homeostasis and aggregation in neurons with toxic Tau overload. Here, I tested this possibility directly, by
showing that expressing either wild type or mutant human Tau in the CNS induced age-dependent neurodegenerative phenotypes including vacuolization in the brain, impaired learning and memory functions, and behavioral deficits in locomotor capabilities in Drosophila. Moreover, all these Tau-induced deficits can be significantly suppressed by overexpression of both wild type and enzyme-dead NMNAT proteins. Importantly, I found that the severity of neurodegenerative phenotypes was correlated with the level of hyperphosphorylated Tau oligomers, and NMNAT overexpression specifically reduced hyperphosphorylated tau oligomers, while increased the level of Tau monomers and high molecular weight aggregates. In addition, I further show that NMNAT protein directly interacted with the Tau oligomers to promote ubiquitylation and clearance of the toxic Tau oligomeric species. The reduction of Tau oligomers was correlated with reduced Caspase 3 activation, suggesting that NMNAT significantly reduces Tau-induced apoptosis and hence rescues tau-induced neurodegenerative phenotypes.

Previous work has shown that overexpression of wild type or mutant Tau causes abnormal vacuolization in the cortex and neuropil structures in flies in an age-dependent manner (Wittmann et al., 2001). Such vacuolization is commonly observed in other neurodegeneration models in Drosophila and correlated with the relative speed and severity of degeneration in flies (Buchanan and Benzer, 1993; Coombe and Heisenberg, 1986; Kretzschmar et al., 1997). However, such degeneration in flies is not coupled with the formation of insoluble neurofibrillary
tangles (Wittmann et al., 2001). It was shown that cholinergic neurons were significantly affected by Tau overexpression (Wittmann et al., 2001), however, in my studies, I observed vacuole formation throughout various parts of the brain when Tau was expressed in the CNS. Therefore, other neuronal populations may also be susceptible to Tau-induced neurodegeneration. In addition to vacuole formation, Tau expression in the CNS resulted in a high level of pTauSer262 accumulation, a phosphorylated form of Tau that is linked with several neurodegenerative conditions (Iijima et al., 2010) throughout the brain. Overexpression of NMNAT proteins (wild type or enzyme-inactive) significantly reduced the size and occurrence of vacuolization throughout the brain. Interestingly, NMNAT expression also affected the levels and distribution of pTauSer262 proteins. Specifically, increased NMNAT levels decreased the overall levels of pTauSer262, reduced the filamentous accumulation of pTauSer262, and increased the colocalization of NMNAT with pTauSer262. These observations indicate that NMNAT protein is recruited to misfolded Tau oligomers and regulate their clearance. The recruitment of NMNAT protein to misfolded protein aggregates has previously been observed in the Drosophila model of SCA1, where NMNAT was recruited together with chaperone Hsp70 to the hATX1-82Q protein aggregates (Zhai et al., 2008b). Therefore, similar neuroprotective mechanism may be at play in the current tauopathy model, where the chaperone function of NMNAT contributes to the regulation of misfolded protein load in the brain.
Learning and memory deficits associated with Tauopathies in *Drosophila* were first reported when Tau was overexpressed in mushroom body neurons, which are known as the centers for olfactory learning and memory (Mershin et al., 2004). In this case, selective Tau overexpression in these neurons affected associative olfactory learning and memory before the onset of apparent neuronal loss. In my study, I expressed Tau proteins in all neurons in the CNS and observed an age-dependent decline of learning and memory indices. In previous studies, such a decline in cognitive functions seems to precede the morphological defects, which only become apparent later with age (Mershin et al., 2004). Interestingly, removing one copy of endogenous NMNAT significantly exacerbated the learning and memory deficits induced by TauR406W expression, suggesting that the level of neuronal NMNAT protein is a critical determinant of the onset and severity of the degeneration induced by mutant Tau. In contrast, higher levels of NMNAT expression rescued the learning and memory and locomotor deficits induced by either wild type or mutant Tau, further suggesting the importance of the neuronal NMNAT levels and a likely protein-protein interaction-mediated neuroprotective mechanism afforded by NMNAT.

Using a *Drosophila* model of tauopathy, I was able to analyze a detailed mechanism of NMNAT’s neuroprotective capacity in ameliorating Tau-induced neurodegeneration. I found that the severity of morphological and behavioral deficits associated with Tau-overexpression is directly correlated with the level of hyperphosphorylated Tau species associated with various diseases (Figure 5.4).
Importantly, NMNAT expression reduced Tau-induced degenerative phenotypes by reducing the level of hyperphosphorylated Tau and promoting the ubiquitination and clearance of toxic Tau oligomers (Figure 5.5).

The key mediator of toxicity in tauopathy is the hyperphosphorylation of Tau. Several studies in animal models with altered kinase or phosphatase activity supported the role of Tau hyperphosphorylation in promoting neurotoxicity (Ahlijanian et al., 2000; Cruz et al., 2003; Jackson et al., 2002; Le Corre et al., 2006; Lucas et al., 2001; Nishimura et al., 2004; Noble et al., 2003; Noble et al., 2005; Steinhilb et al., 2007a; Steinhilb et al., 2007b). For example, when all of the phosphoepitopes created by proline-directed kinases SP/TP sites in Tau were mutated to alanine, the toxicity associated with Tau overexpression was significantly reduced, identifying a positive correlation between increased phosphorylation at disease-associated sites and neurotoxicity (Steinhilb et al., 2007a; Steinhilb et al., 2007b). Here, increased NMNAT levels effectively reduced Tau hyperphosphorylation specifically at sites that are known to be phosphorylated in disease states, including those recognized by AT8 and AT180, and consequently reduced the level of apoptosis and neurodegeneration. One important finding from my study is the specific reduction of ubiquitinated Tau oligomeric species in the range of 150 to 250 kDa and increase of ubiquitinated monomeric Tau and ubiquitinated higher molecular weight oligomeric Tau above 250 kDa in NMNAT overexpressing brains. As NMNAT expression suppressed Tau-induced degeneration, this finding suggest that the mid range (150-250 kDa)
Tau oligomers are likely the species mediating neuronal toxicity, while the monomers and high range (>250 kDa) Tau oligomers are less toxic. This finding is consistent with the emerging notion that high molecular weight (Tau) protein aggregates may be protective when the formation of aggregates can reduce the neuronal load of oligomeric species (Brunden et al., 2008; Iqbal et al., 2005; Iqbal et al., 2008; Takashima, 2008). I showed for the first time a neuroprotective protein NMNAT exerting its protective effects by reducing specifically the toxic Tau oligomers while increasing the less toxic and maybe even protective higher molecular weight Tau species.

Molecular chaperones and their indispensable role in protein folding have been implicated in many neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and Huntington's disease (Carmichael et al., 2000; Ostrerova et al., 1999; Sittler, 2001; Warrick et al., 1999). These stress-regulated proteins prevent improper folding and aggregation of proteins hydrophobic residues of unfolded proteins (Ali et al., 2010; Hartl, 1996; Slavotinek and Biesecker, 2001). The molecular chaperones Hsp70 and Hsp90 were shown to prevent aggregation in a cellular model of tauopathy (Dou et al., 2003). In fact, in Alzheimer's disease brains, the levels of Hsp90 and Hsp70 were shown to be inversely related to the expression of aggregated tau, raising a good possibility that molecular chaperones may protect against tau aggregation and NFT formation (Dou et al., 2003). Previous work uncovered a novel stress-responsive chaperone role of NMNAT, in addition to its role as a housekeeping enzyme in
NAD synthesis (Ali et al.; Zhai et al., 2008a). Similar to Hsp70 and Hsp90 (Dou et al., 2003), NMNAT is also capable of binding to Tau oligomers in our study, and reduce Tau toxic load by promoting ubiquitination and clearance of tau oligomers. Moreover, I also found that reduced endogenous level of NMNAT significantly exacerbated the Tau-induced degeneration, further indicating the critical role of NMNAT in maintaining neuronal integrity and regulating the neuronal misfolded protein load. Interestingly, gene-array studies found that the level of human neuronal NMNAT isoform NMNAT2 was reduced in brain specimens derived from AD patients (https://www.nextbio.com), providing a causative link between reduced NMNAT levels and AD.

In summary, overexpression of human wild type of mutant Tau induced neurodegeneration that can be suppressed by NMNAT expression. NMNAT interacts directly with Tau and modulates neuroprotection in tauopathy in an enzyme-independent manner. Here, I further report a molecular mechanism of Tau-induced degeneration and NMNAT-mediated neuroprotection placing NMNAT in the category of neuronal chaperones that are critical in regulating neuronal protein homeostasis, modulating misfolded protein load, and maintaining neuronal integrity. Understanding the regulation of NMNAT would be interesting and crucial to further explain its role in maintaining proper neuronal health, and would open novel therapeutic windows for this protein in neurodegenerative disease treatment.
7.3 *NMNAT acts as a neuronal maintenance factor by interacting with endogenous proteins*

In wild type Drosophila, NMNAT is localized mainly in the cell body and at the synapse, at high levels (Zhai et al., 2006). Previous work has confirmed that NMNAT can work as a chaperone, independent from its enzyme function (Zhai et al., 2008a). Furthermore, I have already shown that NMNAT is induced upon stress, like other heat shock proteins (Ali et al., 2011). All this evidence categorizes NMNAT as a chaperone that is constitutively expressed and induced by stress, similar to what is seen with Hsp90. The fact that NMNAT has special compartmentalized localization within a neuron coupled with its enzyme-independent chaperone activity raises the possibility that it can have discrete protein binding partners under normal conditions.

Other constitutively expressed chaperones that can be further activated by stress, such as Hsp90, have multiple client proteins that they can bind to and chaperone to make these proteins localize properly or aid in binding other partners (Taipale et al., 2010). In particular, Hsp90 facilitates the maturation of a wide range of proteins that act as signal transducers, including kinases and transcription factors, thereby regulating diverse cellular functions and exerting marked effects on normal biology (Taipale et al., 2010). Several similarities exist between Hsp90 and NMNAT: First, they are both present at high levels, found in many compartments of a cell; Second, both of them have chaperone activity and
can help maintain proper folding of proteins (however, it is unclear if NMNAT can refold misfolded proteins); Third, they are both upregulated by stress conditions in a similar manner. These similarities were the rationale behind investigating the presence of NMNAT's client proteins.

Based on data gathered from immunoprecipitation experiments of NMNAT from wild type adult whole brain lysate, both Brp and alpha-tubulin were shown to interact with NMNAT (Fig. 6.2-6.3). This provides the first insight into a possible mechanism of NMNAT-mediated neuroprotection. Most of the recent attention on NMNAT came from work on the Wld<sup>s</sup> mice, where overexpression of different NMNAT isoforms can delay axon degeneration after injury (Mack et al., 2001). Moreover, the protection availed in these mice comes from a chimeric protein, which allows for higher levels of NMNAT1 to be present (Conforti et al., 2000; Mack et al., 2001). Despite more NMNAT, the Wld<sup>s</sup> mice do not show higher levels of NAD produced at any time (Mack et al., 2001), which draws attention to other functions that NMNAT can perform, if present above a certain threshold. In fact, enzyme-deficient NMNAT can also provide neuroprotection in Drosophila models of SCA1, directing attention to the chaperone function attributing to at least some of this neuroprotective capacity (Zhai et al., 2008b).

In fact, these two binding partners can explain some of the earlier published observations. Loss of NMNAT in photoreceptors causes severe neuronal and synaptic degeneration post development (Zhai et al., 2006). With
activity, the degeneration phenotype is accelerated even more (Zhai et al., 2006). In \textit{nmnat} mutant photoreceptors, numerous cellular structures are disrupted throughout the entire neuron, including rhabdomeres and the presynaptic terminal, and the phenotype becomes progressively more severe with age (Zhai et al., 2006). Interestingly, at the presynaptic terminals, the number of terminals per rhabdomeres is significantly reduced in \textit{nmnat} mutant photoreceptors, showing that active zone stability is drastically affected (Zhai et al., 2006). Here, I show that NMNAT specifically interacts with Brp, the major structural backbone of the T-bar (active zone) structure. In a situation where NMNAT levels are reduced beyond a certain threshold, Brp levels are also drastically reduced at the synapse. Such an interaction can definitely explain a role of NMNAT at stabilizing the synapse. Currently, more efforts are underway to study the possible consequences of NMNAT-Brp interaction, with focus on how this might affect synaptic maintenance.

Earlier work on Wld\textsuperscript{s} mice showed that these mice microtubule acetylation was enhanced in cultured cerebellar granule cells from Wld\textsuperscript{s} mice (Suzuki and Koike, 2007). Acetylation is known to occur in microtubules that have been formed already and is hence used as a marker for stable microtubules. However, this observation was explained through the action of mammalian Sir2-related protein (SIRT2) (Suzuki and Koike, 2007), a tubulin deacetylase belonging to the Sir2-related protein (sirtuin) family of NAD-dependent deacetylases (North et al., 2003), suggested to promote both microtubule hyperacetylation and resistance to
axonal degeneration in these cells. This study qualified SIRT2 as a downstream effector of the protective pathway mediated by Wld<sup>8</sup>. However, my studies here show that NMNAT can directly bind preformed microtubules (Fig. 6.3G), explaining why it is able to pull down both acetylated tubulin and alpha tubulin from wild type brain lysates. Currently, work is under progress to investigate the context under which NMNAT can bind microtubules and that if this binding has any effect on microtubule stability, polymerization or dynamics.

Identification of these binding partners will provide a mechanistic understanding of how NMNAT provides neuroprotection and can help design therapeutic use of this protein to treat neurodegenerative diseases and axonal injuries.

7.4 Summary
The neuroprotective role of NMNAT has been well documented in various model systems. However, there is a lack of mechanistic insights into how this protein offers extended neuroprotection in neurons faced with both internal and external insults. Previous work has solely focused on examining the extent of neuroprotection availed by different isoforms of NMNAT, attributing the protective effects to either differential cellular localization or to the benefits of NAD synthase function of NMNAT to overall cellular health. However, recently a novel enzyme-independent chaperone function was identified for NMNAT that was also shown to contribute to the neuroprotection seen with increasing the levels of the wild-
type protein. Here, I elaborate on this earlier finding, to show that Drosophila NMNAT is a stress-responsive protein and that both the enzyme-dead and wild-type protein can offer neuroprotection in models of tauopathy. The importance of the chaperone function is highlighted in its mechanism to ameliorate the effects of neurodegeneration in Drosophila models of tauopathy, where NMNAT can bind to hyperphosphorylated Tau oligomers and reduce the toxic species via ubiquitination and proteasomal degradation. I have extended my studies to include mammalian CNS-specific NMNAT1 and 2 and show that in a mouse model of Tauopathy, NMNAT2, the predominant brain isoform is significantly downregulated transcriptionally, prior to the onset of neurodegeneration. Furthermore, mammalian NMNAT2 is under the transcriptional regulation of pCREB, which is itself less active in this disease model, hence leading to a reduction in levels of NMNAT2. This is a very novel and interesting finding as it supports the maintenance function of NMNAT2, which has been identified very recently, and ties neurodegeneration to a failure in neuronal maintenance.

My transcriptional analysis reveals that Drosophila NMNAT is upregulated by stress via the direct binding of HSF to its promoter; at the same time, mammalian NMNAT 2 is under the regulation of pCREB, which maintain steady levels of this protein in healthy neurons to perform its maintenance functions (Fig. 7). The discrepancy at the level of Drosophila NMNAT and mammalian NMNAT2 reveals that the only fly isoform of this protein is regulated similar to mammalian NMNAT1, which is seen to be upregulated in cardiac myocytes upon hypoxia.
Figure 7.1 Transcriptional regulation of NMNAT in Drosophila and mammals. In Drosophila, there is one NMNAT gene, under the stress-dependent regulation of HSF. During acute stress conditions, NMNAT is upregulated by direct binding of HSF to a HSE present in its promoter. In mammalian CNS, the two predominant isoforms are NMNAT1 and 2. NMNAT2 is involved in neuronal maintenance and is regulated by pCREB. Neuronal CREB activity is known to be increased by NMDAR-mediated calcium influx through several signaling pathways CREB is generally activated by a variety of signaling cascades including activation through andenylyl cyclase –cAMP-PKA pathway, and direct phosphorylation via activity-dependent increase in CaMKIV. However, in tauopathy, mislocalization of Tau fibrils and oligomers into dendritic spines cause reduction in NMDARs, which could account for a reduction in pCREB levels. Alternatively, AKT activity and pGSK3 levels have also been shown to affect pCREB levels and these signaling cascades are known to be disrupted in tauopathy.
Such a form of regulation is physiologically more relevant in tissues that are more rapidly affected by such acute stress paradigms, such as the heart upon an ischemic episode. Drosophila might be able to bypass such differential regulation by adopting alternative splicing that results in two different isoforms, one acting like NMNAT2, being sensitive to chronic stress such as tauopathy, while the other being maintained at high levels post acute stress regulation (unpublished data).

Collectively, my work uncovers novel insights into the regulation of the neuronal maintenance factor NMNAT; harnessing such regulatory pathways can affect the levels of this protein and can be further used to maximize the neuroprotective capacity of NMNAT. Identifying the endogenous targets of NMNAT will provide possible mechanism of NMNAT-mediated neuroprotection.
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