DOES Hsp27 PROTECT CORTICAL NEURONS AGAINST AMYLOID?

MICHAEL T.C. KING

Does Hsp27 Protect Cortical Neurons Against Amyloid?

By

© Michael T. C. King (Honours)

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Abstract

Neurofibrillary tangles (NFTs) and plaques are considered to be hallmarks of Alzheimer's disease (AD), although it is still not clear whether these aggregates contribute to disease progress or whether they might even play an initial protective role. A number of studies have shown that the small heat shock protein Hsp27 can afford protection against oxidative stress, growth factor withdrawal or excitotoxicity in neuronal cell types. A moderate preconditioning heat shock (HS) is sufficient to upregulate levels of Hsp27 in cells, and the degree of protection correlates with the level of expression of Hsp27. There are reports of increased Hsp27 in AD brains and accumulation of Hsps in plaques, NFTs and Lewy bodies. Whether this represents a potentially protective response to the stress or is part of the disease process is not known. My hypothesis is that increased expression of Hsp27 can promote survival and stabilize the axonal cytoskeleton resulting in maintenance of neurite growth and axonal transport in the face of stressors such as amyloid. My specific aim was to determine whether Hsp27 is important for neuronal survival and neurite growth in primary cortical neurons in the presence or absence of ß-amyloid peptides.

Cultures of primary rat cortical neurons were subjected to HS in an effort to upregulate endogenous Hsp27, while in other experiments Hsp27 was overexpressed in cortical neurons by nucleofection. Efforts to upregulate endogenous Hsp27 in cortical neurons were unsuccessful, despite the fact that the neurons do express heat shock factor-1 (Hsf1) and increase activation of Hsf1 in response to heat stress. In fact, it was seen that HS resulted in a reduction in neuronal survival, although previous data from our lab and the literature suggest that a thermal stress can provide

protection against a more lethal subsequent stress. Consequently, I introduced a GFP-Hsp27 fusion protein at the time of plating and examined the potential effects of exogenous Hsp27 on neurite survival and growth using morphological and biochemical analyses.

My results suggest that the presence of Hsp27 in cortical neurons provides them with a level of protection against ß-amyloid. After treatment with $A\beta_{1-42}$, cells with Hsp27 appeared healthier and had greater survival than those without. Interestingly, $A\beta_{25-35}$ was not shown to be toxic to these neurons, whereas it has been previously shown to be toxic in a variety of experimental paradigms. Further, my data indicate that cells expressing Hsp27 have greater total neurite growth compared to the empty vector alone. Knowing that phosphorylation affects Hsp27 activity, I also investigated the effects of Hsp27Δ on survival and growth. Hsp27Δ is a mutated form of Hsp27 lacking the S15 phosphorylation site. My data show that HspΔ has similar effects on neurite outgrowth and survival as Hsp27WT, providing important preliminary data for future studies investigating the importance of phosphorylation in Hsp27 activity.

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Aß	amyloid beta
AD	alzheimer's disease
ADDL	amyloid derived diffusible ligand
AICD	amyloid intracellular domain
Akt	protein kinase B
APH-1	anterior pharnyx defective 1
APOE	apolipoprotein E
APOE-ε4	apolipoprotein E allele type 4
APP	amyloid precursor protein
CNS	central nervous system
DDT	dithithreitol
DIV	days *in vitro*
DRG	entorhinal cortex
EC	dorsal root ganglia
ECL	enzymatic chemiluminescence
EGFP	enhanced green fluorescent protein
EMEM	eagles minimum essential media
EV	empty vector
FCS	fetal calf serum
FTDP	frontotemporal dementia with parkinsonism
GFAP	glial fibrillary acid protein
Grp78	glucose regulatory protein or HspA5
HBSS	hanks balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
HS	heat stress
Hsf1	heat shock factor – 1
Hsp	heat shock protein
Hsp27Δ	heat shock protein27 delta
JNK	jun-N-terminal kinase
kDa	kilo Dalton
MAP	microtubule associated protein
MAPK	mitogen activated protein kinase
MAPKAP	mitogen-activated protein kinase-activated protein
MAPKAP-K2	mitogen-activated protein kinase-activated protein kinase-2
MT	microtubule
MTS	(3-(4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl)-2-(4-sulfonphenyl)-2H-tetrazolium compound
NB	neurobasal
NFT	neurofibrillary tangles

NGF	nerve growth factor
NIH	national institute of health
P1	post-natal day one
p38	p38 mitogen activated protein
PBS	phosphate buffered saline
PC12	pheochromocytoma cell line
Pen-2	presenilin 2 secretase
PF	protofibrils
PI 3-K	phosphatidylinositol 3-kinase
PKCδ	protein kinase C (delta isoform)
PS1	presenilin 1 secretase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sHsp	small heat shock protein
TBS	tris-buffered saline
TBST	tris-buffered saline with 0.5% Tween
Tg	transgenic
WT	wild type

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Chapter 1

Introduction

Alzheimer's Disease (AD) is a neurodegenerative disease characterized by premature neuronal death in the presence of neurofibrillary tangles (NFTs), and extracellular neuritic plaques. NFTs are aggregates of hyperphosphorylated tau protein, whereas neuritic plaques are insoluble extracellular aggregates of Aβ, among other components. The amyloid hypothesis asserts that apoptosis is caused by the destabilization of the cytoskeleton in the presence of extracellular amyloid plaques (Mattson, 2004, Price *et al.*, 1998, Song *et al.* 2002, Tanzi and Bertram, 2001). A great deal of investigation has been directed towards the amyloid hypothesis since the discovery of Aβ plaques, but the direct mechanism by which amyloid acts to cause cell death has yet to be fully elucidated.

Heat Shock Proteins (Hsps) are a small family of highly conserved proteins whose expression can be induced by stressors, such as heavy metals, elevated temperatures and alcohols (Hightower, 1991). Hsps have been shown to be protective, demonstrate chaperone activity and enhance microtubule (MT) growth and stabilization. In particular, the small heat shock protein (sHsp) Hsp27 has been shown to protect cells by inhibiting caspases and promoting Akt activity, a pro-survival signal (Mearow *et al.*, 2002, Dodge *et al.*, 2006). In other words, cells can achieve tolerance to larger more toxic stress after a sub-lethal stress (Hightower, 1991, Mailhos *et al.*, 1994). For example, cells over-expressing Hsp27 were more resistant to nerve growth factor (NGF) withdrawal, and

displayed less caspase-9 activation than those not over-expressing Hsp27. Similarly, Hsp27 has been shown to reduce apoptosis through cytochrome C and fas pathways (Patel *et al.*, 2005) and thus it is not surprising that Hsp27 has been shown to attenuate caspase-3 activation as well (Garrido *et al.* 1999, Mearow *et al.*, 2002). In addition, Hsp27 appeared to interact with Akt and sustain its activity, demonstrating that Hsp27 plays a role in protecting neurons by inhibiting apoptosis and promoting survival (Mearow *et al.*, 2002). Hsps have been shown to act as molecular chaperones that refold stress-denatured proteins and shuttle damaged proteins to the degradation pathway (Becker and Craig, 1994, Parsell and Lindquist, 1993, Rogalla *et al.*, 1999), two important processes considering that neurons are post mitotic cells that are unable to avoid deleterious protein aggregation by division. Furthermore, it is possible that Hsp27's protective ability may be independent of signaling pathways or chaperone activity. For example, Hsp27 has been shown to interact with the cytoskeleton (Huot *et al.*, 1996, Lavoie *et al.*, 1993, Miron *et al.*, 1991) and some have inferred that its protective effects are due to its ability to act as an actin capping protein (Benndorf *et al.*, 1994, Mounier and Arrigo, 2002). Given Hsp27's protective characteristics, it is possible that Hsp27 could protect CNS neurons from cell death caused by protein aggregation and axon destabilization, the central molecular pathologies of Alzheimer's disease.

1.1 The Amyloid Hypothesis

Amyloid protein (Aβ) is strongly implicated in AD, as described by the amyloid cascade hypothesis (Fig. 1.1). The amyloid hypothesis, although somewhat controversial,

asserts that mutated Aβ accumulates in protein aggregates to form plaques that lead to NFTs. NFTs obstruct axonal transport and causes a destabilization of the cytoskeleton which leads to cell death and ultimately dementia. Despite the plethora of research on AD and Aβ (Pub Med search of "Alzheimer's" AND "Amyloid" reveals 17355 results - http://www.ncbi.nlm.nih.gov.qe2a-proxy.mun.ca/sites/entrez) a complete description of the events that cause cell death is unknown.

Formation of toxic Aβ begins with proteolytic processing of the transmembrane protein, amyloid precursor protein (APP). APP is a ubiquitously expressed protein whose function remains unclear. It is thought that APP may inhibit serine proteases and play a role in cell adhesion and neuroprotection (Maccioni *et al.*, 2001) but, of more relevance to this thesis, it has been suggested that APP is involved in vesicle trafficking in the axon (Zheng *et al.*, 1998), an interesting insight considering that a suspected pathology of AD is a failure of axonal stability. Interestingly, APP can be increased by stress since its promoter contains a heat shock element (Johnson *et al.*, 1997). Furthermore, APP interacts with actin, tubulin, neurofilaments (Cottrell *et al.*, 2005) and with kinesin I, a key protein in microtubule transport (Kamal *et al.*, 2000). This may implicate APP in axonal maintenance and suggest that a failure of APP processing may lead to abnormal axonal function and possibly degeneration.

APP is cleaved by the multimeric gamma secretase protease complex, APH-1 (Anterior Pharnyx Defective 1), Nicastrin and the more commonly known presenilin 1 and 2 secretases (PS1, Pen-2, Fig. 1.1A). Upon cleavage, the C-terminus remains as an amyloid intracellular domain (AICD), whose function is unknown, while the N-terminus

segment is released extracellularly as Aβ (Fig. 1.2B). However, it may be important to note that transfection of certain forms of AICD have been shown to induce cell death in HeLa and pheochromocytoma (PC12) cells (Kinoshita *et al.*, 2002)

It is thought that an error in APP or its secretases leads to the production of erroneous $A\beta_{1-40}$ or $A\beta_{1-42}$ that fibrilize in a series of steps to form Aβ aggregates that subsequently lead to neuronal plaques, tangles and AD (Fig. 1.1, Hardy and Selkoe, 2002). Genetic analysis reveals eleven pathogenic mutations in the APP, all of which are missense mutations located within or in close proximity to the region encoding Aβ (Tanzi and Bertram, 2001). $A\beta_{1-42}$ deposits early in the disease and is considered to be the most toxic species despite the fact that it accounts for only 10% of total secreted Aβ (Price *et al.*, 1998). Furthermore, $A\beta_{1-42}$ has a higher susceptibility to fibrilize and self aggregate (Harper *et al.*, 1999, Pike *et al.*, 1995).

Some suggest that Aβ binds the cell surface (Venkitaramani *et al.*, 2007) allowing for interaction with NMDA receptors that would influence memory and synaptic plasticity, while others (Heredia *et al.*, 2004) suggest that Aβ can bind integrins, leading to the activation of cellular pathways that promote cytoskeletal remodeling. Furthermore, p75 has been shown to bind $A\beta_{1-40}$ (Yaar *et al.*, 1997), $A\beta_{25-35}$ (Kuner *et al.*, 1998) and $A\beta_{1-42}$ (Sotthibundhu *et al.*, 2008). Binding was shown to mediate cell death in neurons and as well in other experimental designs (Dechant and Barde, 2002, Sotthibundhu *et al.*, 2008, Troy *et al.*, 2002).

Another explanation of Aβ induced toxicity is that Aβ aggregates form pores in the cellular membrane allowing for a rapid influx of Ca^{++} and subsequent activation of

apoptosis (Kawahara and Kuroda, 2000, Resende *et al.*, 2007). In these experiments, $A\beta_{1-42}$ and $A\beta_{25-35}$ were the predominantly toxic peptides in comparison to other forms of Aβ such as $A\beta_{1-40}$ and $A\beta_{1-28}$. Similarly, Pike *et al.* (1993) showed that incubation of $A\beta_{1-42}$ and $A\beta_{25-35}$, but not $A\beta_{1-28}$, resulted in neurotoxicity. On the other hand, some researchers suggest that Aβ formation is not necessary for AD progression (Hardy and Selkoe, 2002) and that tangles alone can produce AD dementia. This theory is largely supported through the study of missense tau mutations (Hutton *et al.*, 1998, Poorkaj *et al.*, 1998) in other AD-like degenerative diseases that have NFT pathology and subsequent degradation such as frontotemporal dementia with parkinsonism (FTDP). Correlative studies show that severity of plaque formation does not correlate well with cognitive impairment (Whalen *et al.*, 2005). However, in tangle diseases, like FTDP, there is no accumulation of Aβ as seen in AD. In addition, the first APP mutant mouse generated presented with age dependent neuronal plaques, dystrophic neurites, hippocampus atrophy, a loss in synaptic density, but did not display NFTs (Masliah *et al.*, 1996). Further, the gene encoding tau is not genetically linked to AD, demonstrating that tangle pathology can be mutually exclusive of Aβ deposits (Hardy and Selkoe, 2002). Advocates of the Aβ hypothesis suggest that tau is downstream of Aβ accumulation. For example, it was shown that intra- and extracellular accumulation of Aβ preceded NFT pathology in the 3xTg mouse, an AD mouse model (Oddo *et al.*, 2004). When mice were treated with anti-Aβ antibodies, plaques and NFTs disappeared, but when treatment was halted the deposits returned, followed by NFTs, showing that amyloid acts in a hierarchical manner to

produce NFTs (Oddo *et al.*, 2004). Although the Aβ hypothesis is controversial, it remains popular.

1.2 Aβ Toxicity Prior to Aggregation

The idea that Aβ plaques lie at the core of AD progression is a widely held view. Numerous studies have shown a correlation between aggregation state of Aβ and neurotoxicity (Busciglio *et al.*, 1995, Mattson, 2004, Pike *et al.*, 1993). For example, Aβ fibril-induced toxicity has been associated with neuronal pathology, such as axon and dendrite dystrophy and this toxicity was shown to be fibril dependent (Lorenzo and Yankner, 1994). However, the existence of soluble species that precede fibrilized Aβ, such as Aβ derived diffusible ligands (ADDLs) and protofibrils (PFs), have been a recent area of interest (Kim *et al.*, 2003, Klein, 2002). ADDLs are small globular structures approximately 5 nm in diameter whereas PFs are curvilinear shaped structures 25-200 nm in diameter. PFs have been shown to alter action potential and membrane depolarization (Hartley *et al.*, 1999) whereas ADDLs have been shown to cause an attenuation of long term potentiation (Lambert *et al.*, 1998). Not surprisingly, both PFs and ADDLs were shown to induce neuronal cell death. Similarly, the small soluble fragment $A\beta_{25-35}$ induces toxicity in the pheochromocytoma (PC12) cell line, a well characterized neuronal model, indicating that $A\beta_{25-35}$ may be the biologically toxic fragment of the full length Aβ peptide (Yankner *et al.*, 1990). Furthermore, exposure to the $A\beta_{25-35}$ fragment has been shown to result in a significant reduction in hippocampal and cortical viability (Pike *et al.*, 1993). Thus, it is thought that more soluble oligomers are responsible for the

neurodegenerative changes that occur in AD (Costantini *et al.*, 2005). Neurodegenerative changes that lead to dystrophic axons include inhibition of actin polymerization, cytoskeletal disruptions and disorganization, all cytoskeletal effects in a selective neuronal population that have been attributed to Aβ induced cell death.

1.3 Amyloid and the Cytoskeleton

A typical neuron is characterized by long thin extensions that can account for the largest proportion cell volume. As a result, an efficient transport system (i.e. protein, vesicle, and organelle transport) is necessary for viability. Any disruption or interference in this system can jeopardize growth, connections and cell viability. MTs and related proteins form a trafficking system that span from the cell body to the leading edge, where growth cones and synapse form, two integral constituents of a healthy neuron. Given that NFTs are aggregates of microtubule associated tau, it is likely that NFTs are playing a role in degradation by interfering with axonal transport system. For example, it has been shown *in vitro* that Aβ treatment can induce tau phosphorylation on epitopes the same as those that result in pathological hyperphosphorylated tau, followed by gradual loss of neuritic processes. Furthermore, phosphorylation resulted in a decrease in microtubule associated protein (MAP) binding that was reversible by a dephosphorylation agent, alkaline phosphatase (Busciglio *et al.*, 1995). These data imply that Aβ treatment leads to tau phosphorylation, disassociation of MAPs and possibly MT instability (Michaelis *et al.*, 2005). In a similar study a MT stabilizing drug, taxol, was shown to afford a 90% reduction in Aβ-induced cell death (Michaelis *et al.*, 2005), while taxol alone (without

Aβ) did not demonstrate an increase in survival or reduction in apoptosis. This suggests that taxol's protective effect was due to stabilization of the axon and not mediated by anti-apoptotic signaling. Therefore, it may be reasonable to postulate that agents that stabilize MTs can provide protection, independent of cell signaling. For example, Hsp27 has been shown to bind hyperphosphorylated tau and promote its dephosphorylation and degradation, thereby promoting cellular survival (Shimura *et al.*, 2004).

On the other hand, Aβ has been shown to induce actin stress fiber formation, leading to disorganization and aberrant actin polymerization, resulting in clogging of the axon and eventual degeneration (Song *et al.*, 2002). The actin cytoskeleton forms the basis for synaptic plasticity of the neuron and is dynamic in the order of seconds (Shea *et al.*, 1997, Toni *et al.*, 1999) such that, when disrupted it can lead to growth cone collapse and degeneration of the dendrites (Meberg and Bamburg, 2000). In theory, these data provide promising research for sHsps that are known to stabilize actin and promote survival by acting as an actin capping protein.

Extracellular Aβ aggregates have been shown to occur prior to NFTs but recently it has been suggested that intracellular accumulation of Aβ is a feature of early AD pathogenesis (Alafuzoff *et al.*, 2008, D'Andrea *et al.*, 2001) and that a significant intracellular accumulation could lead to axonal abnormalities, degeneration and neuronal death that precedes extracellular plaques (Takahashi *et al.*, 2008). For example, transgenic mice demonstrate behavioral abnormalities prior to the accumulation of extracellular plaques (Chapman *et al.*, 1999) but coincident with the intracellular accumulation of Aβ (Wirths *et al.*, 2008). In the 3xTg mouse, containing mutations in

presenilin, tau and APP, intraneuronal accumulation of Aβ preceded NFT pathology and Aβ plaques (Oddo *et al.*, 2004). Furthermore, intracellular Aβ has been shown to down-regulate PI-3 kinase/Akt survival pathways (Magrane *et al.*, 2005) which resulted in neuronal death. In addition, sHsps were upregulated in response to Aβ but were insufficient to prevent cell death. Interestingly, a viral over-expression of Hsp70 has been shown to rescue cells from intraneuronal Aβ induced toxicity (Magrane *et al.*, 2004), showing that an exogenous over-expression of a sHsp was better able to protect than an endogenous one.

1.4 Alternatives to the Amyloid Hypothesis

Some researchers have gone even earlier into the origin of AD pathology and suggested an early pathogenesis in the synapse. Hardy and Selkoe (2002) suggested that transgenic mice undergo synaptic dysfunction before the appearance of plaque aggregation and that synaptic dysfunction is the earliest pathology of the disease. For example, AD mice bearing the V717F mutation demonstrated a 40% loss in basal synaptic transmission of the hippocampus between the age of one to four months, prior to the appearance of plaques. This is further supported by electron micrograph analysis by Davies *et al.* (1987) who reported a 25-35% reduction in synaptic density of cortical neurons biopsied from clinical AD patients. Furthermore, AD patients' cognitive decline is correlated with synaptic loss (Scheff *et al.*, 1990, Terry *et al.*, 1991). Certainly, synaptic loss is heavily implicated as an early feature of AD (Cotman and Anderson, 2000, Deshpande *et al.*, 2006, Townsend *et al.*, 2006) and neurons exposed to Aβ have

shown reduced synaptophysin immunoreactivity, suggesting a disruption or loss in synaptic contacts (Busciglio *et al.*, 2005). Interestingly, Hsp27 has been shown to be located in the synaptic cleft and neuronal plaques of AD brains (Stege *et al.*, 1999) and it is possible that Hsp27 may be present in the cleft to provide protection, possibly through chaperone activity.

Stress related genes and oxidative stress are shown to increase in the Alzheimer brain and it is thought that neurons may become vulnerable and die as they are exposed to increasing levels of oxidative stress. For example, it has been shown that $A\beta_{1-42}$ can generate hydrogen peroxide, an oxidative species that is known to cause lipid peroxidation that can interfere with glucose and glutamine transporters (Mattson, 2004). Interestingly, an upregulation of Hsp70 and Hsp27 can protect neuronal cells against superoxide dismutase mutant-induced cell death (Patel *et al.*, 2005) and oxidative stress has been shown to have an affect on important metabolic processes such as a decrease in cytochrome C oxidase, pyruvate dehydrogenase and the α-ketoglutarate deyhydrogenase complex (Mattson, 2004), all of which have been shown to be decreased in the brains of AD patients (Blass, 2001). Hsp27 expression is increased in AD brains (Renkawek *et al.*, 1993, 1994) but whether this augmented expression is a response to the aging process is unknown. However, given the known protective effects of Hsp27, it is possible that Hsp27 may be upregulated to reduce stress associated with AD and aging.

Apolipoprotein E (APOE), a serum protein of four allele types (ε1-4) whose function is to regulate cholesterol, has been implicated in AD, particularly APOE-ε4 (Price *et al.* 1998). The occurrences of AD patients having the APOE-ε4 allele is

significantly greater than the control population and is highly associated with Aβ plaque levels in the brain (Bales *et al.*, 1997). However, the number of ε4 alleles better predicts the age onset than the risk of getting AD. In other words, ε4 predicts when, not whether, the disease will occur (Meyer *et al.*, 1998) and as a result is not considered as a causative gene for AD.

1.5 Alzheimer's Disease and Hsp27

AD is a neurodegenerative disease characterized by pathology within a selective neuronal population that become dysfunctional and die (Mattson, 2004). Neurodegeneration in AD has been well characterized (Braak *et al.*, 2006) and is correlated with NFTs and Aß deposition (Bierer *et al.*, 1995). Initially, cell loss includes magnocellular neurons, of the nucleus basalis of Meynert, that are located in the basal forebrain. These neurons are of particular importance as they are a major source of cholinergic projections to the neocortex (Muth *et al.*, 2009). Following, lesions occur in the limbic system, particularly in the entorhinal cortex (EC) and hippocampus. In the final stages of AD, the neocortical lesions become more predominant and cognitive function is heavily impaired (Braak *et al.*, 2006). Clinical symptoms parallel structural degeneration as memory loss is followed by weakening of higher cognitive processes such as episodic memory and spatial orientation (Jellinger *et al.*, 1990). Ensuing degeneration in the transentorhinal cortex, basal forebrain and entorhinal cortex lead to a progressive reduction in choline acetyltransferase and cholinergic neuronal loss (Davies and Maloney, 1976), consequently having a dramatic impact on cognition (Cassel *et al.*,

2008). Thus, the strong association between declining cholinergic markers and overall cognitive impairment (Pappas et al., 2000, Frolich, 2002) may explain the transition from mild cognitive impairment to full blown AD. Not surprisingly, this relationship is the guiding principle behind cholinomimetic therapy, the prominent drug treatment for dementia.

Neurons themselves are particularly vulnerable to protein aggregation because they are post mitotic cells that are unable to divide to avoid the deleterious effects of misfolded proteins that accumulate during aging (Muchowski and Wacker, 2005). Hsps have been implicated in protein aggregation, axonal growth and transport and destabilization of neurofilaments, actin and MTs. For example, missense mutations of small Hsps such as Hsp27, 22 and 8 are associated with peripheral nervous system neuropathies, particularly the axonal form of Charcot Marie Tooth disease and distal hereditary motor neuropathy (Evgrafov *et al.*, 2004, Irobi *et al.*, 2004). Likewise, *in vitro* it has been seen that growth and complexity in dorsal root ganglion neurons (DRGs) is dramatically reduced following decreased expression of Hsp27 by siRNA oligonucleotides (Williams *et al.*, 2006). These reports imply that an interruption of Hsp expression can lead to distinct phenotypes related to growth and axonal pathology. Furthermore, mutations in both neurofilament and Hsp27 cause similar phenotypes such as protein aggregation and disruption of neurofilaments resulting in pathological axonal transport (Ackerley *et al.* 2006, Evgrafov *et al.*, 2004, Perez-Olle *et al.* 2004). Therefore, Hsps may play a role for growth, protein folding, axonal stabilization and transport, all of which have been shown to become pathological in Alzheimer's disease. Perhaps it is the

lack of expression of Hsp27 that could leave neurons vulnerable to degradation in the presence of stress. Knock down of Hsp70 and 90 by siRNA has been shown to lead to insoluble aggregates of tau that inhibits tau association with MTs (Dou *et al.* 2003).

Hsps are divided into 5 classes based on their molecular weight Hsp 100, 90, 70, 60 and the sHsps, including Hsp27, also known as Hsp25 and HspB1 (Ingolia *et al.*, 1982). The defining quality of Hsps is their ability to ameliorate cell insult following an initial sub-lethal stress. It has been shown that the degree of initial stress is correlated with their expression and that their ability to protect cells under a larger more significant cellular insult is independent of the nature of the stress. For example, a mild heat stress produced an equivalent ability to protect cells when under another subsequent more intense heat or ischemic stress (Amin *et al.*, 1995). Furthermore, Hsp27 transgenic (tg) mice have shown remarkable resistance to kainate induced seizures, demonstrating less severe seizures and lower mortality rate in comparison to non-tg littermates (Akbar *et al.*, 2003). Morphological analysis revealed a reduction in atrophic changes such as cell shrinkage and chromatin condensation. Similarly, it was shown that Hsp27 in tg mice could protect motor neurons after a neonatal sciatic nerve crush where these mice later demonstrated significantly higher muscle mass, motor units, and muscle force contraction in comparison to wild type (WT) litter mates (Sharp *et al.*, 2006). Whether Hsp27 prevented early neuronal death or reversed muscle damage is unclear but these data suggest that Hsp27 can prevent neuronal death and consequently, protect muscle function.

It should be noted that Hsps are not constitutively expressed in all cell types and may require stress for their expression, while some sHsps, despite their nomenclature, are

not necessarily upregulated by heat stress but are known to upregulate in response to chemical stressors (Taylor and Benjamin, 2005). For example, αA-crystallin is almost exclusively expressed in the eye lens whereas αβ-crystallin is expressed in eye, muscle cells and astrocytes (Kato *et al.*, 1991). Furthermore, Hsp70 is expressed at low levels in the brain under normal conditions but is highly inducible in response to injury such as hyperthermia, ischemia or seizure (Armstrong *et al.* 1996, Manzerra *et al.*, 1997, Krueger *et al.* 1999), whereas cortical neurons lack Hsp27 (Bechtold and Brown, 2003).

1.6 Hsp27 Structure

Hsp27's secondary structure is highly conserved with other Hsps, for example αβ-crystallin, suggesting that Hsps could be involved in normal cell processes that are common between cell types and organisms. For example, Hsps are found in both mycobacterium and human. Hsp's chaperone activity is known to maintain conformation for proteins involved in intracellular transport, cytoskeletal architecture, translation regulation and homeostasis (Arrigo *et al.*, 2007), suggesting a role in regular cell maintenance. It has previously been shown in primary sensory neurons that Hsp27 is present in neurites, branch points and growth cones and is colocalized with actin, tubulin and neurofilament in DRG neurons, implying that Hsp27 is involved in normal growth of the axon. Furthermore, alterations in the phosphorylation status of Hsp27 results in aberrant neuritic growth, however, the exact nature of this role in not clearly understood (Williams *et al.*, 2005).

Hsp27 can be post-translationally modified by phosphorylation via mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP-K2), on three residues in the human – S15, S78 and S82 and two residues in the rodent – S15 and S86 (Gusev *et al.*, 2002). The S15 phosphorylation site is located in the so-called WDPF domain, a conserved region, at the N terminus while the other phosphorylation sites are located just prior to the highly conserved α-crystallin domain (Fig. 1.3). At the C terminus is a variable, flexible and motile tail whose function remains unknown (Gusev *et al.*, 2002, Sugiyama *et al.* 2000). Hsps form large oligomeric aggregates that can be dynamically modified following stress and subsequent activation of MAPK pathways (Lelj-Garolla and Mauk, 2005). However, it should be noted that certain isoforms of protein kinase C, namely PKCδ (Thr 505), can phosphorylate Hsp27 (Takai *et al.*, 2007). Interestingly, PKCδ phosphorylation has been shown to reduce Aβ production by altering APP processing (Gabuzda *et al.*, 1993). The MAP-kinase pathway, including p38 MAPK (p38 mitogen activated protein kinase) and MAPKAP-K2, is activated in response to stress to modify Hsp activity.

Hsp27 normally exists in a large oligomeric structure made of twenty four monomers forming a structure with a molecular mass of 700 kDa but upon phosphorylation Hsp27 can modify its structure (Kim *et al.*, 1998) to form small dimers and naturally, its function is also affected. For example, phosphorylation has been shown to affect chaperone activity (Gusev *et al.*, 2002) and rapid remodeling of the actin cytoskeleton (Guay *et al.*, 1997). The effect of phosphorylation on Hsp27's structure and function has been investigated but remains controversial.

1.7 Phosphorylation and Hsp27's Interactions With the Cytoskeleton

Hsp27s chaperone activity and interactions with the cytoskeleton is heavily influenced by phosphorylation (Lambert *et al.*, 1999). In unstressed cells, Hsp27 exists as complex oligomeric structures, but under stress Hsp27 is phosphorylated and reduced to smaller dimers and monomers (Lambert *et al.*, 1999). Phosphorylation modifies its ability to bind actin and affect its polymerization (Benndorf *et al.*, 1994, Guay *et al.*, 1997). Cell free *in vitro* studies suggest that Hsp27 binds and stabilizes actin filaments by binding to the fast growing plus end of actin filaments, acting as an actin capping protein (Fig. 1.4) inhibiting polymerization. However, upon phosphorylation, Hsp27 is modified into smaller structures and its actin capping ability becomes curtailed, thus promoting polymerization. It is thought that large non-phosphorylated oligomeric Hsp27 stabilize actin via actin capping and some have suggested smaller partially phosphorylated Hsp27 oligomers bind MTs and prevent their degradation under stress (Fig. 1.5). The exact nature of Hsp27/actin interaction is not completely known but it appears that post-translational modification of Hsp27 regulates dynamic modeling of the cytoskeleton in response to stress.

sHsps interact with intermediate filaments such as vimentin, glial fibrillary acidic protein (GFAP) and neurofilaments to maintain their integrity and it appears that these interactions are phosphorylation dependent. Hsp27 has been shown to regulate interactions and assembly of both GFAP and vimentin. Some have proposed that Hsp27 can prevent deleterious filament aggregation by regulating filament-filament interactions (Perng *et al.* 1999). This was supported by Vicart *et al.* (1998) who reported a mutation

in sHsps can lead to intermediate filament aggregation. Others suggest that Hsp27 binds vimentin to maintain a soluble pool of subunits that maintain network integrity under stressful conditions (Lee *et al.*, 2005). Furthermore, Hsp27 and the related αβ-crystallin have demonstrated a similar effect in maintaining GFAP networks (Perng *et al.*, 1999), suggesting a general role of Hsp involvement in neuro- and intermediate filament network stability. Recently, our lab has shown a co-localization of tubulin and Hsp27 in neurites from adult DRG neurons (Williams *et al.*, 2005) and it has been shown elsewhere that Hsp27 can interact with MTs in muscle cells and some cell lines (Hargis *et al.*, 2004, Hino *et al.*, 2000). Thus, Hsp27 has been inferred not only to stabilize actin but neurofilaments, intermediate filaments, MTs and tubulin.

1.8 Summary

Hsp27 has been implicated in AD. Hsp27 has been found in amyloid plaques, the synapse and colocalized with cytoskeletal elements (i.e. neurofilament, intermediate filaments, MAPs and transport proteins such as kinesin) that are responsible for the integrity of the axon; the axon being particularly important to neuronal survival. This suggests that Hsp27 may be interacting with the cytoskeleton and playing a role in normal cell functions, where a failure of these functions could lead to AD pathology. Hsp27 exhibits anti-apoptotic and pro-survival activity, suggesting a role in regulating cell death. Further, Hsp27 has been implicated in normal axonal growth, transport and chaperone activity indicating that an error in these processes could jeopardize neuronal viability. Chaperone activity is particularly important because neurons are post mitotic

cells that can not avoid protein aggregation via cell division. The presence of Hsp27 in Aβ plaques may indicate a failed attempt to rescue neurons, but whether this is part of the disease process is unknown. Recently, Hsp 70 and 90 have been shown to inhibit the early stages of $A\beta_{1-42}$ aggregation *in vitro* (Evans *et al.*, 2006) and this action may be chaperone based. Given the protective effects of Hsp27 and its ability to upregulate under stress, it is reasonable to think that an over-expression of Hsp27 may be able to provide protection against Aβ induced toxicity.

1.9 Hypothesis

My hypothesis is that increased expression of Hsp27 can promote survival and stabilize the axonal cytoskeleton resulting in maintenance of neurite growth and axonal transport in the face of stressors such as amyloid.

1.10 Objectives

- Aim#1. To determine Hsp27 expression and upregulation in cortical neurons.
- Aim#2. To investigate whether Hsp27 is important for neuronal survival in the presence or absence of ß-amyloid peptides.
- Aim#3. To explore the effect of Hsp27 on neurite length in cortical neurons and the effects of phosphorylation in neurite outgrowth.

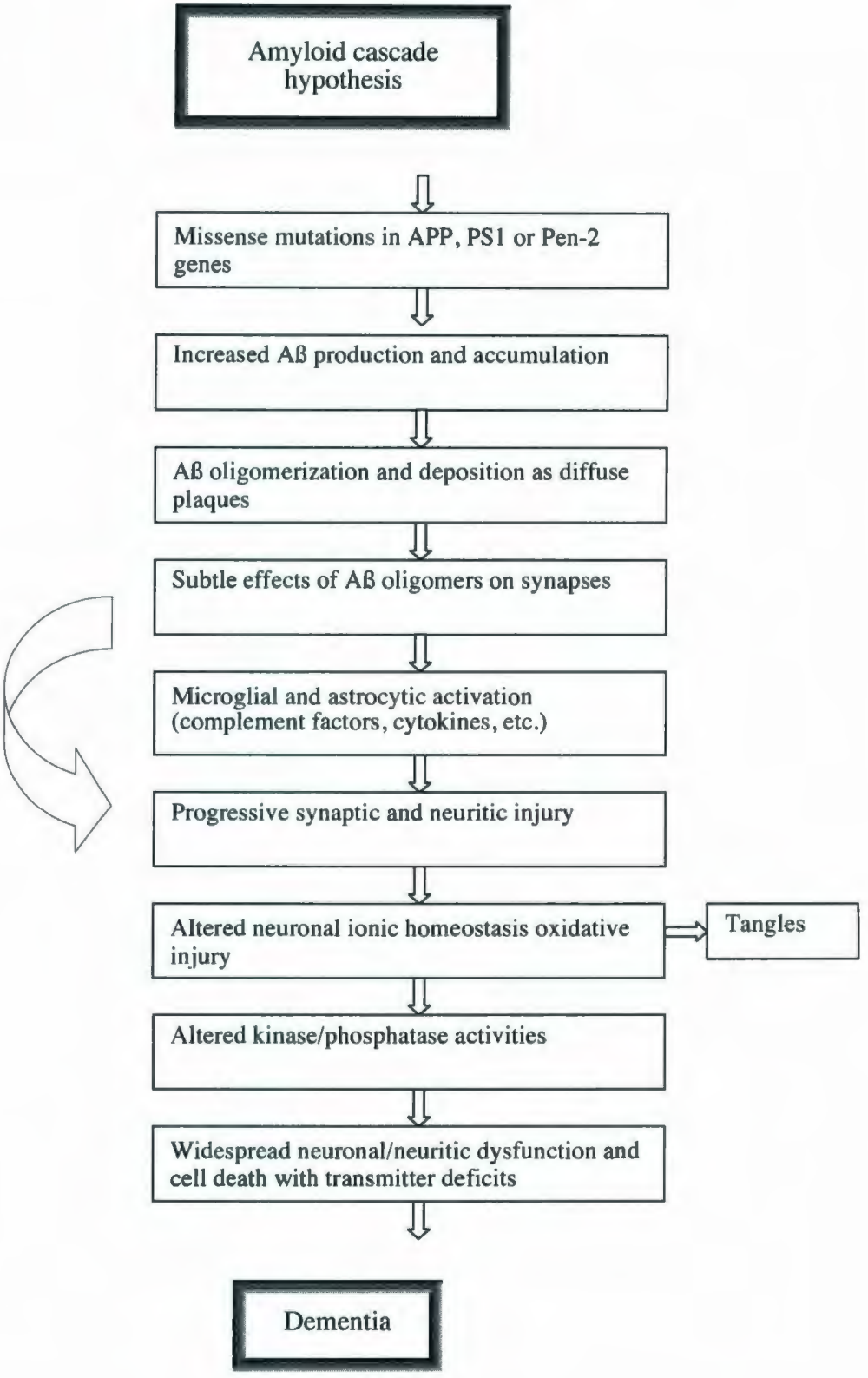
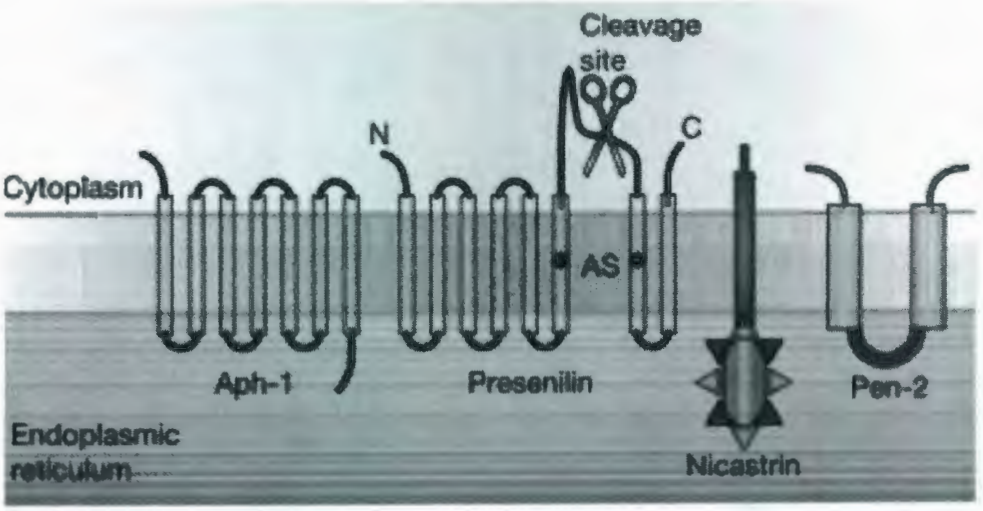


Figure 1.1. Proposed Amyloid hypothesis (Adapted from Hardy and Selkoe, 2002).

A



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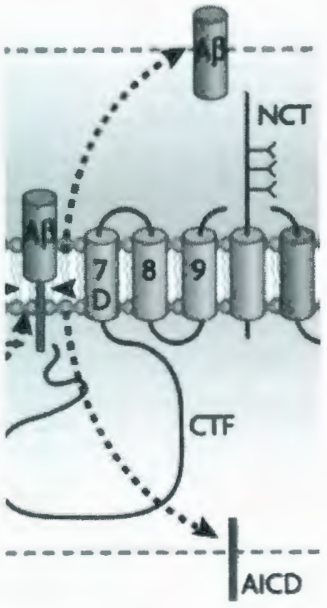


Figure 1.2. Schematic representation of APP processing by the gamma secretase complex. (A) The gamma secretase complex (B) cleaves APP into an intracellular (AICD) and extracellular fragment. The purpose AICD is unknown whereas, according to the Amyloid hypothesis, $A\beta_{1-40}$ and $A\beta_{1-42}$ aggregate and become toxic (Copyrights for figure obtained from Nature, Mattson, 2004).

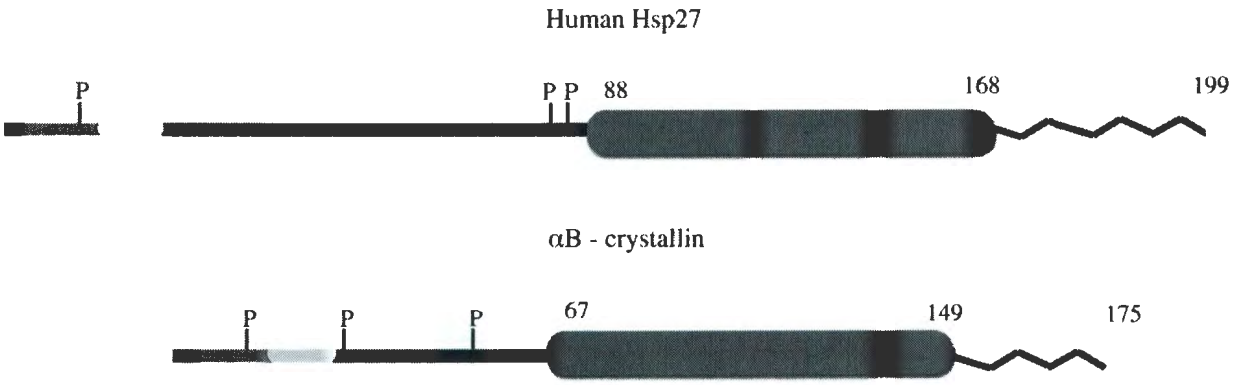


Figure 1.3. Schematic representation of Hsp27 and the related αβ-crystallin. (Adapted from Gusev *et al.*, 2002).

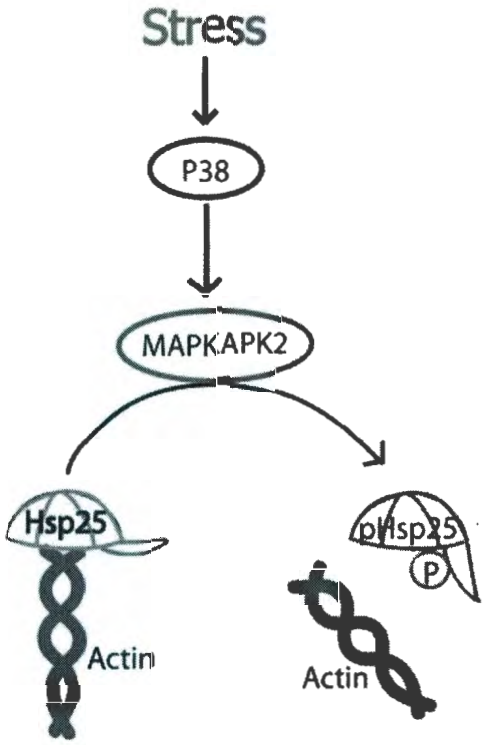


Figure 1.4. Hsp27 binds the growing end of actin, reducing its polymerization. Under stress Hsp27 is phosphorylated, via the MAPKAP-K2 pathway, and demonstrates reduced ability to bind actin, thus increasing polymerization.

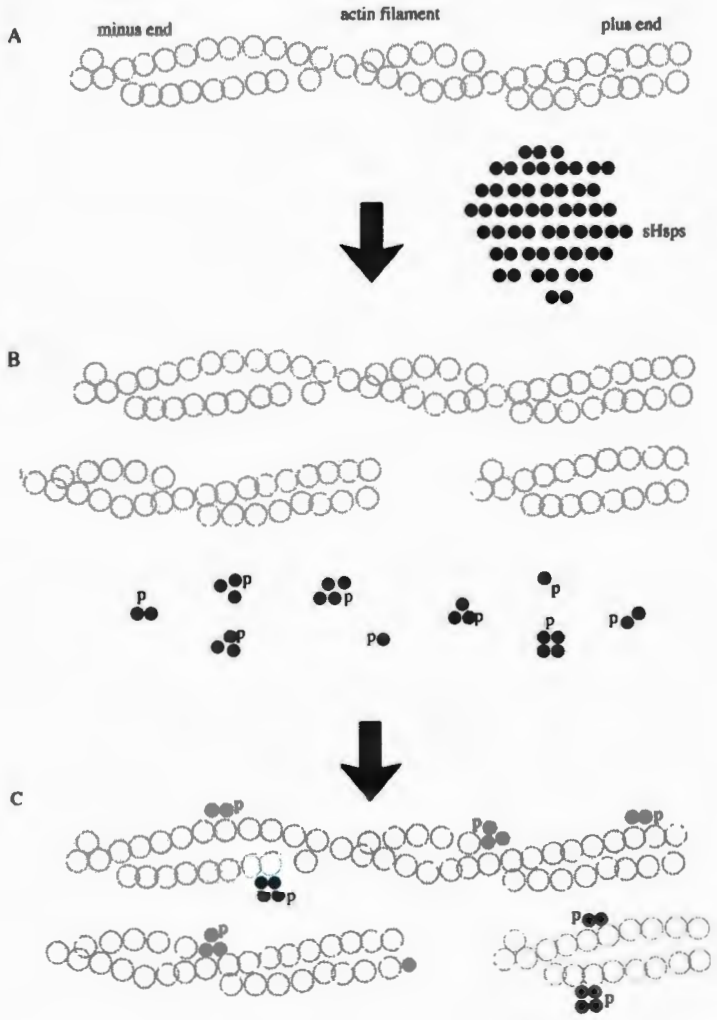


Figure 1.5. Schematic representation of Hsp27's phosphorylation dependent interaction with actin. (A) In unstressed cells, Hsp27 exists as a large oligomeric structure. However, after stress and subsequent phosphorylation, Hsp27 is reduced to monomers dimers and oligomers (B). Phosphorylated products bind MTs whereas non-phosphorylated monomers bind to the fast growing end (C) (Adapted from Mounier and Arrigo, 2002).

Chapter 2

Materials and Methods

2.1 Neuronal Cultures

Brains were removed from post natal day one (P1) Sprague Dawley rats supplied by Memorial University of Newfoundland Animal Care Facility. Animals were sacrificed by rapid decapitation prior to tissue removal and cortex and/or hippocampi were isolated in cold Hanks Balanced Salt Solution (HBSS, 1 mM, Invitrogen, Burlington, ON, Canada). Subsequently the tissue was immersed in 37 °C (HBSS, Invitrogen, Burlington, ON, Canada) containing 0.125% Trypsin (Invitrogen, Burlington, ON, Canada) for 15 min. Trypsin solution was removed and tissue was rinsed with 37 °C HBSS. Soybean Trypsin inhibitor (2 mg/ml, pH to 7.2 using 1N NaOH, Invitrogen, Burlington, ON, Canada) was added and incubated for 5 min at 37 °C to inactivate remaining trypsin. Cells were then rinsed and triturated in dissection media [Eagles minimum essential media (EMEM, Invitrogen, Burlington, ON, Canada), supplemented with 10% fetal calf serum (FCS), 10 mM Sodium Bicarbonate and 20 mM Potassium Chloride] using a pre-wetted 5 ml pasteur pipette. Trituration occurred in 3 steps of 5 triturations each, removing suspended cells after each step. Cells were then washed from debris with dissection media until clean, isolated by centrifugation for 5 min at 225 rcf and counted using a haemocytometer. Three wash steps with warm dissection media were performed in order to remove non-cell tissue and aggregates of dead cells. Cells were counted and plated on poly-D-lysine (1 μg/ml, coated 12-well plates, Falcon, VWR, Mississauga, ON,

Canada) for Western blot analysis or 16-well glass slides (LabTek, VWR, Mississauga, ON, Canada) for neurite growth and survival analysis. Cells were plated in serum-free Neurobasal medium (NB, Invitrogen, Burlington, ON, Canada) supplemented with 100 U/ml penicillin/streptomycin, 2% B-27 supplement and 1 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Invitrogen, Burlington, ON, Canada). Typically, 1.5 x 10^6 cells/ml were plated for protein collection, whereas 2.0 x 10^5 cells/ml were plated for immunocytochemical experiments. Two days after plating, cell culture media was supplemented to 10 mM cytosine arabinoside and at this time, experimental conditions were treated with ß-amyloid peptides (25-35, 1-42, rPeptide, Bogart, GA, USA) or their reverse peptide control (35-25, rPeptide, Bogart, GA, USA). All experimental manipulations were carried out after 2 days *in vitro* (div) in order to allow neurons to recover from the dissociation process. Cell cultures were heat shocked for 15 min at 45°C and subsequently incubated for varying periods of recovery time (3, 6, 12, 24 and 48 hr).

2.2 Transfection

Cells were transfected at the time of plating with Amaxa nucleofector (Amaxa biosystems, Walkersville, MD, USA) according to manufacturer's protocol. Three constructs were employed: i) pMaxEGFP (enhanced green fluorescent protein), control empty vector (EV), ii) EGFP-Hsp27 fusion protein (WT), which have previously been shown to be functional in PC12 cells (Mearow *et al.* 2002) and iii) EGFP-Hsp27Δ. After trituration, cells were counted using a haemocytometer, and 5.5 x 10^6 cells were

pelleted by centrifugation for 5 min at 225 rcf and resuspended in 100 µl of Amaxa transfection solution containing 82 µl of Amaxa nucleofector solution and 18 µl of supplement. Cell suspensions were mixed with 5 µg of DNA, added to Amaxa cuvette and transfected using the G-13 program for hippocampal or cortical neurons. Immediately after transfection, cells were resuspended in EMEM plating media and allowed to recover in an incubator for 30 min at 5% CO_2 and 37 °C. Finally, cells were plated in serum-free NB supplemented with 100 U/ml penicillin/streptomycin, 2% B-27 supplement and 1 mM HEPES (Invitrogen, Burlington, ON, Canada).

2.3 Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min then permeabilized with 0.1% Triton-X and blocked with 10% goat serum. Primary antibodies employed included polyclonal Hsp27 (1:200, Stressgen,Victoria, BC, Canada),and beta III tubulin (1:100, Sigma Chemicals, St. Louis, MO, USA). Cells were incubated with primary Ab for 16-20 hr at 4°C, rinsed with PBS and incubated with Cy2 and Cy3 conjugated secondary antibodies (1:100, Jackson Laboratories, Bar Harbor, ME, USA) for 1 to 2 hr. Cells were finally rinsed in TBS/TBST (Tris-buffered saline/Tris-buffered saline with 0.5% Tween) and coverslipped with 50% glycerol. Images were acquired in each of two channels (Cy2, and Cy3) using confocal scanning microscopy with sequential Z-stage scanning (Olympus Flouview 300 microscope).

2.4 Biochemical Analysis

Neurons were collected by aspirating the medium, adding 200 µl ice cold TBS 2 mM sodium orthovanadate solution and scraping neurons off the plate surface using a rubber scraper. Cells were pelleted for 5 min at 1310 rcf, suspended in ice cold protein lysis buffer (1% NP40, 10% glycerol in TBS plus protease inhibitor tablets, 1 mM sodium vanadate and sodium fluoride, all obtained from Sigma Chemicals, St. Louis, MO, USA) and stored in -20°C until Western blot analysis. All protease inhibitor tablets were obtained from Roche Diagnostics (Laval, Quebec, Canada). Lysates were thawed on ice and subsequently centrifuged for 10 min at 8160 rcf and the supernatants were used to determine protein concentrations using the BSA protein assay kit (Pierce Chemicals, Rockford, IL, USA). Sample buffer [10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1 M dithithreitol (DDT), 0.005% bromophenol blue] was added to 30 µg of protein lysate, boiled for 5 min and then separated by SDS-PAGE for ~1.5 hr at 110V, 40 mA on a 10% acrylamide gel. Electrophoresis was run in electrophoresis buffer [25 mM Tris base, 250 mM glycine (pH 8.3), 0.1% SDS)]. Subsequently, separated proteins were transferred onto nitrocellulose membrane in a transfer tank at 100 V, 200 mA, 4 °C for 55 min. Each blot was then stained with Ponceau Red to ensure equal protein; Ponceau Red was washed off with TBS and blots were blocked with 3% milk/TBS prior to antibody incubation to avoid non-specific binding. Nitrocellulose blots were incubated at 4°C overnight with the following antibodies: Hsp27 (1:1000, Hsp27-Ab 801, Stressgen, Victoria, BC, Canada), phosphospecific Akt (1:500), phospho-p38 MAPK (1:500), Akt (1:1000), p38 MAPK (1:1000) (NEB/Cell Signaling, Beverley, MA), and

actin (1:1000, Sigma Chemical, St. Louis, MO, USA). Blots were then washed in TBST (3 x 15 min) and signals were detected using horseradish peroxidase (HRP) labeled secondary antibodies in 3% powdered milk (1:5000, Chemicon, Temecula, CA, USA) and ECL reagent (NEN, Boston, MA, USA). Films (Cronex MRF clear base, Agfa Corp., Greenville, SC, USA) were developed, subjected to densitometry using ImageJ (National Institute of Health (NIH), Bethesda, Maryland, USA) and prepared using Adobe Photoshop graphics software (San Jose, CA, USA).

2.5 Growth Analysis

Confocal laser scanning microscopy was used to obtain images and neurons were then traced using a semi-automated tracing plug-in NeuronJ within Image J software (NIH, Research Services Branch, Bethesda, Maryland, USA). Total neurite length is the sum of all axons and dendritic processes from a single neuron measured in μm and each data point represents at least 45 neurons from multiple experimental replicates.

2.6 Peptide Preparation

Ultra pure $A\beta_{1-42}$ peptide was prepared by manufacturer's (rPeptide) instructions. White lyophilized powder was re-suspended in 1% NH_4OH at a concentration of 1 mg/ml and sonicated until dissolved. Amyloid peptide solution was then aliquoted, stored at –80 °C and thawed on ice before use. Peptide was not incubated for fibril formation prior to treatment. Peptide (or reverse 35-25 scrambled peptide) were added to cultures on 2 div

and incubated for varying periods of time (4, 6, 12, 24, and 48 hr). Final peptide concentrations of $A\beta_{1-42}$ and $A\beta_{25-35}$ were 5 μM and 25 μM, respectively.

2.7 Statistical Analysis

Statistical analysis was performed in GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA, USA) with significance determined by one-way ANOVA testing. Post-hoc t-tests were carried out after ANOVA testing to directly compare two groups. Significance was determined when $p < 0.05$ and figures display mean values $\pm$ SEM.

2.8 Survival Assays

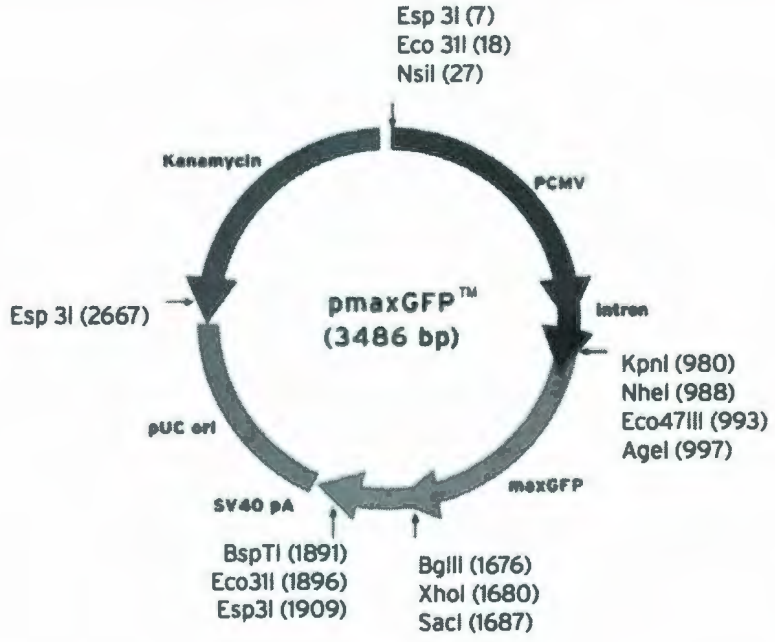
MTS . An initial measurement of survival was assessed using the MTS (3-(4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl)-2-(4-sulfonphenyl)-2H-tetrazolium compound) assay. The MTS assay is an indirect assessment of survival where MTS is converted into formazan by mitochondria, producing a color change detectable at 490nm. Decreased absorbance is an indication of decrease in mitochondrial metabolism, which has been correlated with decreased cell survival (Mosman *et al.*, 1983).

Trypan Blue. The Trypan blue exclusion dye assay was used to investigate the effects of Aß types on cell survival at the individual cell level. Trypan blue dye is excluded from cells with intact membranes but cells that take up the dye are considered to have damaged membranes causing increased cellular permeability. 5 μl of 0.4 % trypan was added to each chamber of a 16 well slide and incubated for 30 min with 5% CO_2. Wells were then twice rinsed with PBS and photographed using a light microscope

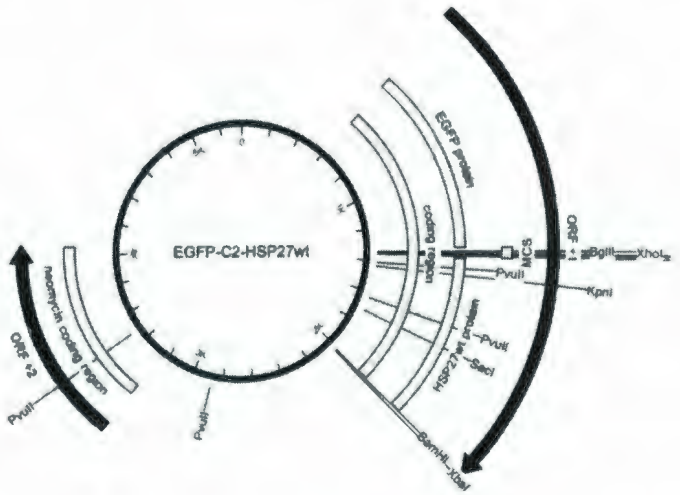
and camera. Survival was assessed over several fields of view and neurons positive for trypan dye were counted relative to total number of cells.

2.9 Constructs

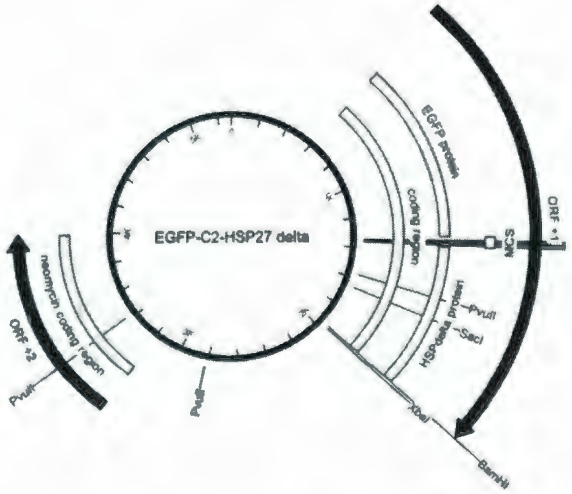
Three constructs were employed for transfection of cortical neurons: EGFP, EGFP – Hsp27WT, EGFP – Hsp27Delta (Δ). Hsp27 cDNA for Hamster was generously donated by Dr. Jacques Landry (Universite de Laval, Quebec City), sub-cloned into pEGFP-C2 vector (Clontech Laboratories, Palo Alto, CA), the empty vector (EV), and was transfected into neurons at the time of plating using Amaxa. The deletion construct was mutated, sequenced and cloned into the same pEGFP-C2 construct.



EGFP



EGFP – Hsp27WT



EGFP – Hsp27Δ

Figure 2.1. Schematic representations of EGFP, Hsp27WT and Hsp27Δ construct.

Chapter 3

Results[1]

A number of studies have shown that the small heat shock protein Hsp27 can afford protection against oxidative stress, growth factor withdrawal or excitotoxicity. A moderate preconditioning heat stress (HS) is sufficient to upregulate levels of Hsp27 in cells, and the degree of protection correlates with the level of expression of Hsp27 (Mearow *et al.* 2002). For example, cell types previously studied in our lab (DRG neurons and PC12 cells) have been shown to upregulate Hsp27 expression in response to heat shock (Mearow *et al.*, 2002, 15 min at 45°C). However, previous research has shown that rat cortical neurons do not normally operate to express detectable levels of endogenous Hsp27, although others have shown Hsp27 in non-neuronal cells (Bechtold and Brown, 2003). Consequently, my first aim was to investigate Hsp27 expression in untreated and heat shocked cortical cell cultures.

3.1 Endogenous Hsp27 expression is not detected in cortical neurons

In order to examine the expression of Hsp27 in cortical neurons in culture, cells were plated on 12 well plates or 16 well slides. Some cells were subjected to heat shock to evaluate whether this could upregulate Hsp27. Protein lysates were extracted for Western blot analyses. Previous studies in our lab demonstrate that Hsp27 is robustly

[1] These results have been included in a recent publication. King M, Nafar F, Clarke J and Mearow M (*In Press*) The small heat shock protein, Hsp27, protects cortical neurons against the toxic effects of β-amyloid. Journal of Neuroscience Research.

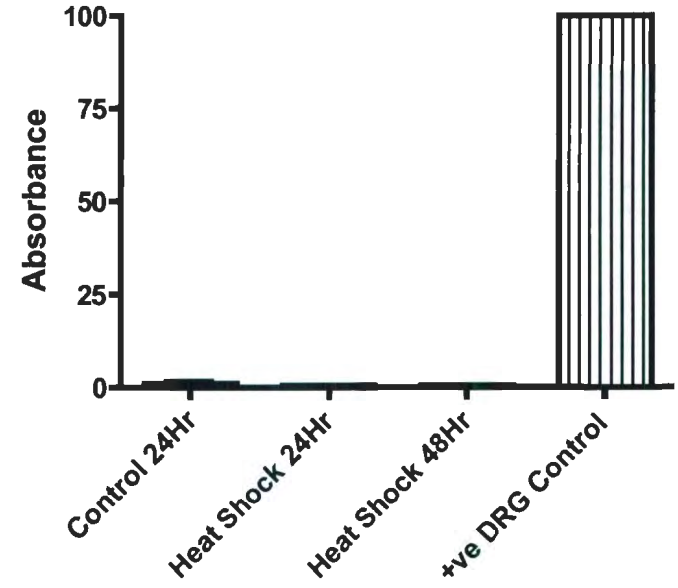
expressed in DRG neurons (Williams *et al.*, 2005) and thus a DRG sample was used as a positive control. Fig. 3.1 shows that Hsp27 is strongly present in positive control sample but not in control or heat shocked cortical cells. This result confirms previous research that Hsp27 is not present in cortical neurons. We also assessed the effect of HS on the viability of the cultures (Fig. 3.2). It was seen that HS resulted in degenerative processes and cell death after 24 and 48 hr.

Consequently, it was necessary to find another means to express Hsp27 in cortical neurons to test the hypothesis of my project. Thus, I attempted to transfect neurons with EGFP-Hsp27WT cDNA using Amaxa transfection. This procedure results in 30-40% transfection as assessed visually in Fig. 3.3A, B, C and Western blot in Fig. 3D demonstrating that Hsp27 is expressed in transfected neurons but not in EV conditions. Note that because several different conditions were run on this particular gel, the pertinent lanes have been selected for presentation. The complete blot is attached in the appendices (Fig. 5.1). Successful expression of EGFP-Hsp27Wt cDNA in preliminary experiments allowed me to investigate Hsp27's effects on survival in cortical neurons.

3.2 $A\beta_{1-42}$ but not the small $A\beta_{25-35}$ fragment has deleterious effects on neurons

Given the variety of Aβ species used in AD research, it was pertinent to examine the effects of multiple Aβ types on cortical neurons *in vivo*. $A\beta_{1-42}$ and $A\beta_{25-35}$ have shown to be toxic in several experimental paradigms (Kawahara and Kuroda, 2000, Kim *et al.*, 2003, Pike *et al.*, 1995, Price *et al.*, 1998, Resende *et al.*, 2007). Therefore, prior to my investigation of aim #2, I conducted preliminary experiments that investigated the effects

Figure 3.1. Western blot and accompanying densitometry shows undetectable levels of Hsp27 in untransfected cells. Cortical neurons were plated on poly-D-lysine coated dishes and allowed to mature for two days in culture. Cultures were heat shocked for 15 min at 45°C and allowed to recover for 24 and 48 hr. Hsp27 is not detectable at 24 or 48 hrs post HS. All data in figure is displayed as mean ± SEM. *** $p < 0.0001$ n=2 (ANOVA).


100
75
50
25
0
Absorbance
Control 24Hr
Heat Shock 24Hr
Heat Shock 48Hr
+ve DRG Control

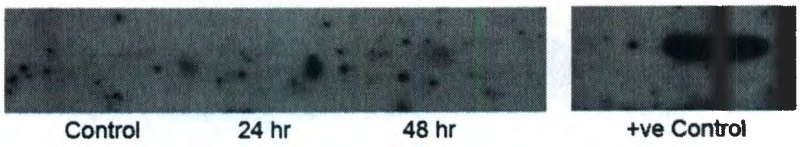
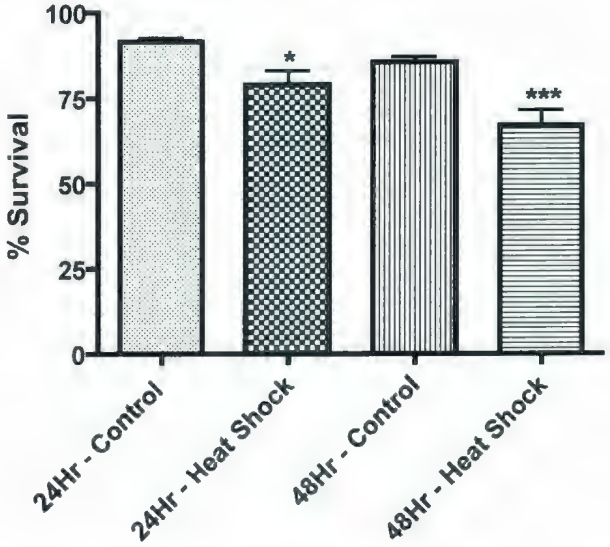

Control
24 hr
48 hr
+ve Control

Figure 3.2. Heat shock reduces survival of cortical neurons as assessed by the trypan blue assay. In other cell systems, heat stress can be used to upregulate Hsps that can provide some level of protection against subsequent stressors. However, my study shows that heat shock results in neuronal death. Cultures were subjected to a mild heat stress of 45 °C for 15 min and allowed to recover for either 24 or 48 hr before analysis. Survival was assessed using trypan blue staining and neurons positive for trypan dye were counted relative to total number of cells. Control conditions did not receive a heat shock (A) and phase micrographs provide an example of normal and heat shock conditions at 24 and 48 hr (B). Images a and c depict control neurons for 24 and 48 hr respectively and images b and d show neurons treated with HS. Note that cells in heat shock conditions show clear degeneration, indicated by arrows. All data in figure is displayed as mean ± SEM. *$p < 0.001$, ***$p<0.0001$ (ANOVA) n=3.

A


100
75
50
25
0
% Survival
*
***
24Hr - Control
24Hr - Heat Shock
48Hr - Control
48Hr - Heat Shock

B

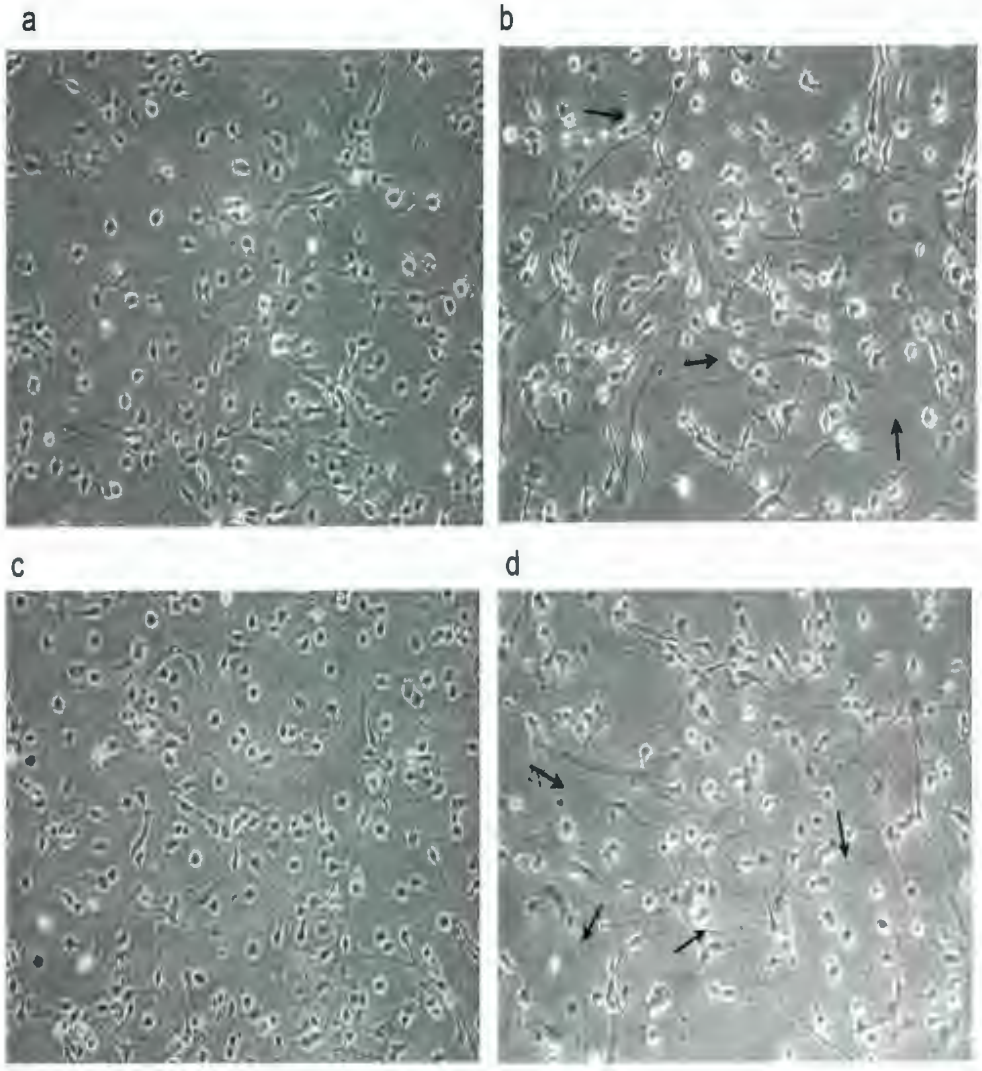
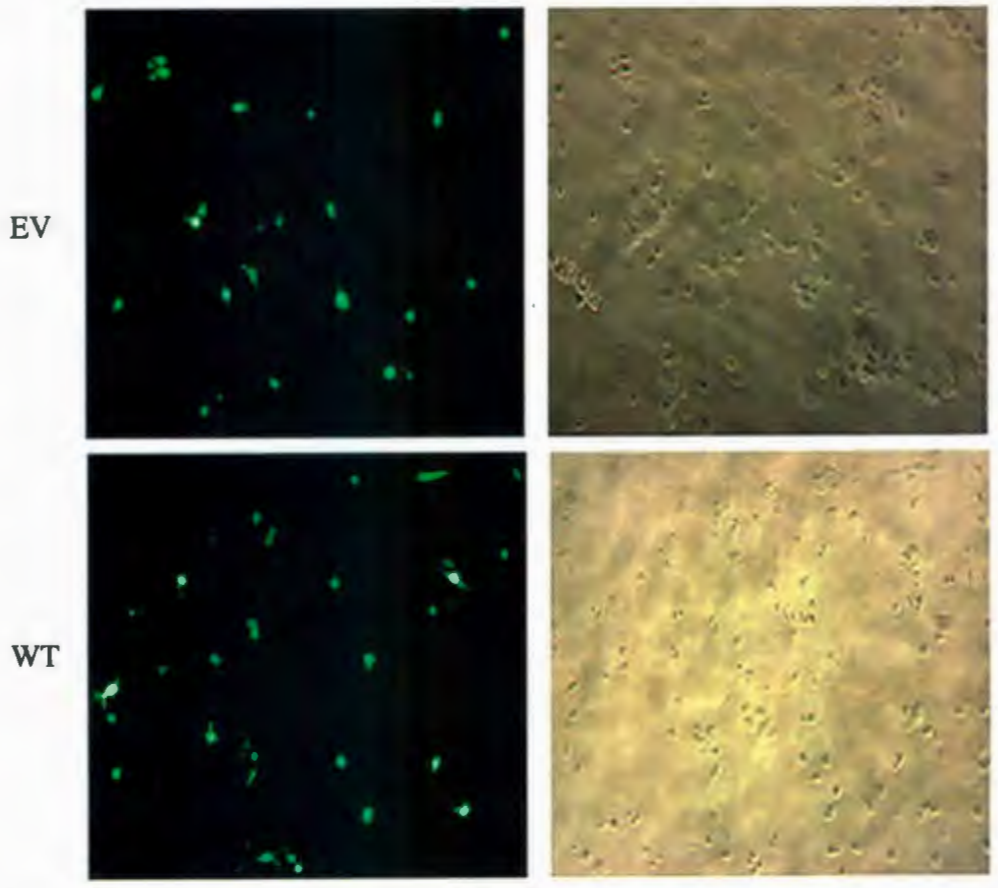
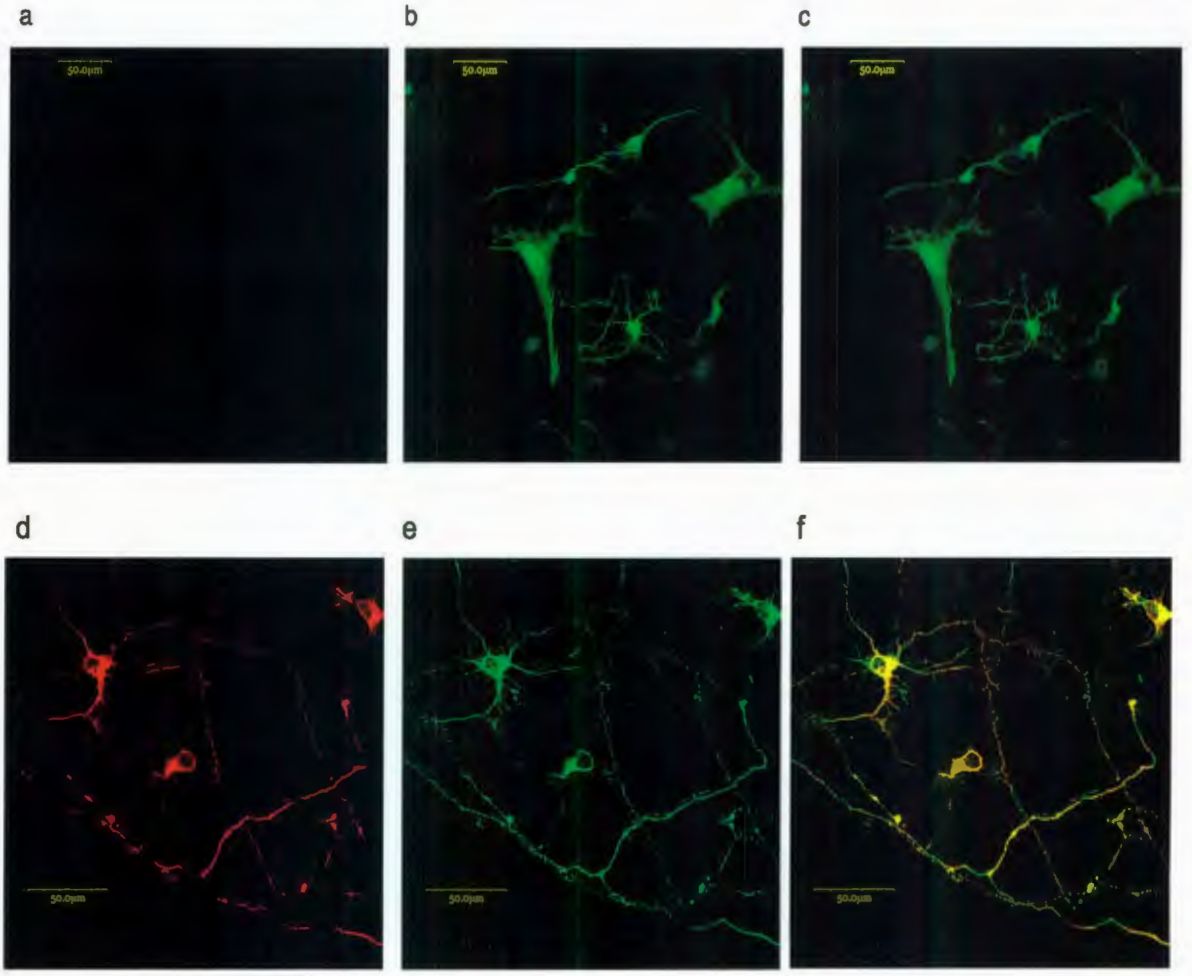

a
b
c
d

Figure 3.3. Neurons can be successfully transfected with vectors. Neurons were plated on poly-D-lysine coated 12 wells or 16 well slides and transfected at the time of plating with either EV or WT cDNA using Amaxa nucleofector. Neurons were either collected for Western blot or immunostained at 48 hr with an antibody directed against Hsp27. Confocal and phase contrast images show that approximately 30-40% of neurons were successfully transfected (A). Hsp27-GFP is detectable by immunohistochemistry but not detectable in untransfected cells (B). Neurons in panels' a-c show transfection with the EV (b, green) but display no detectable levels of endogenous Hsp27 (a). Merged image (c) shows that only EV is present. Neurons in panels d-f show co-localization of the Hsp27WT cDNA (e, green) with immunostaining for Hsp27 (d, red). Merged image showing co-localization can be seen in (f). (C) Shows a typical neuron positive for the neuronal specific marker beta III tubulin (a, red), Hsp27 (b, green) and a merged image showing co-localization of tubulin and Hsp27 (c). Western blot and accompanying densitometry (D) demonstrates successful Hsp27-GFP expression. Blot was probed with antibody against Hsp, which recognizes the fusion protein at 54 kDa, only in the WT lanes. The apparent band in the EV lanes is an artifact of cutting the blot. All data in figure is displayed as mean ± SEM. ***$p<0.0001$ n=2 (ANOVA)

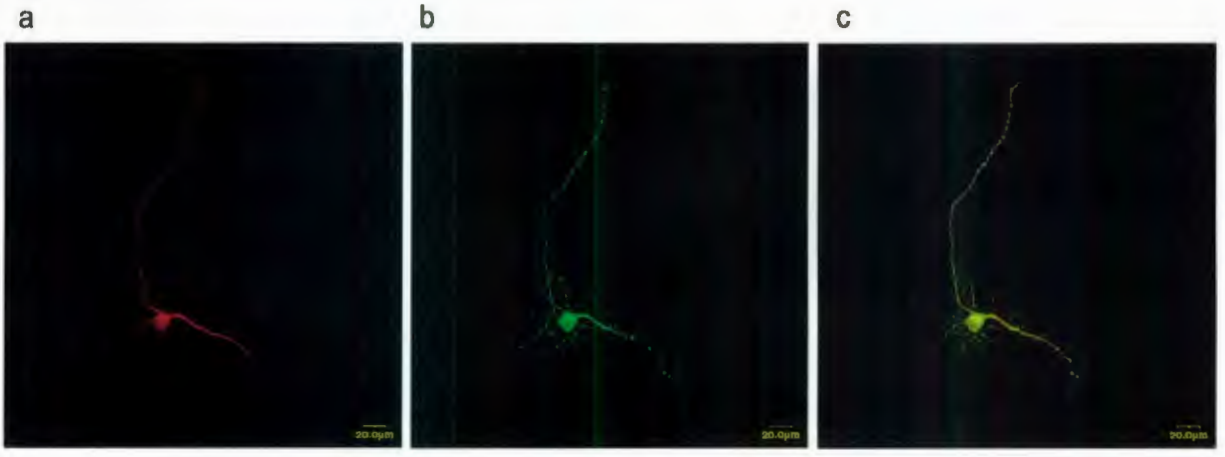
A



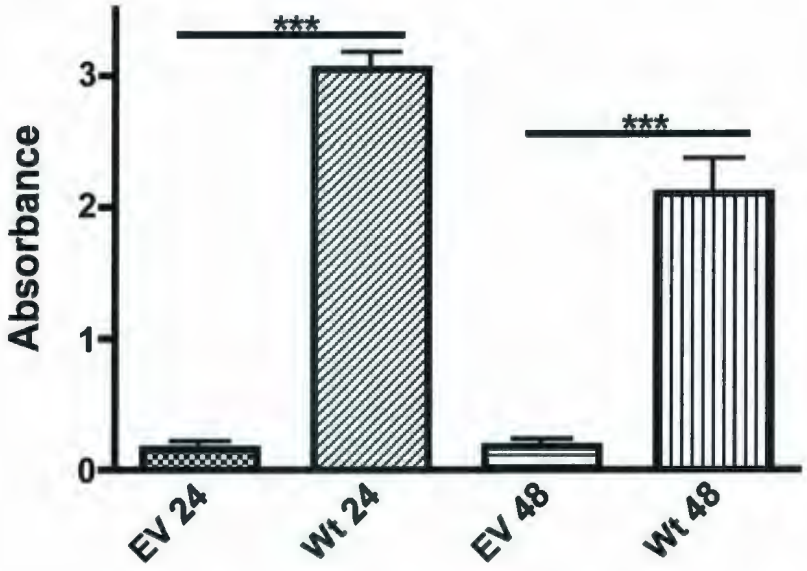
B

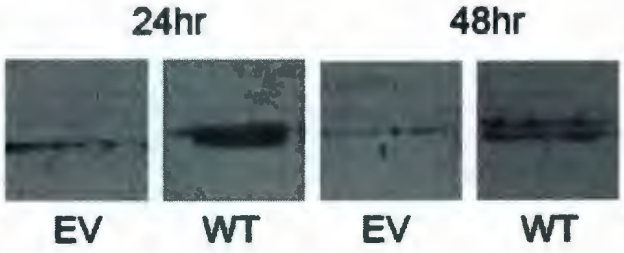


C



D





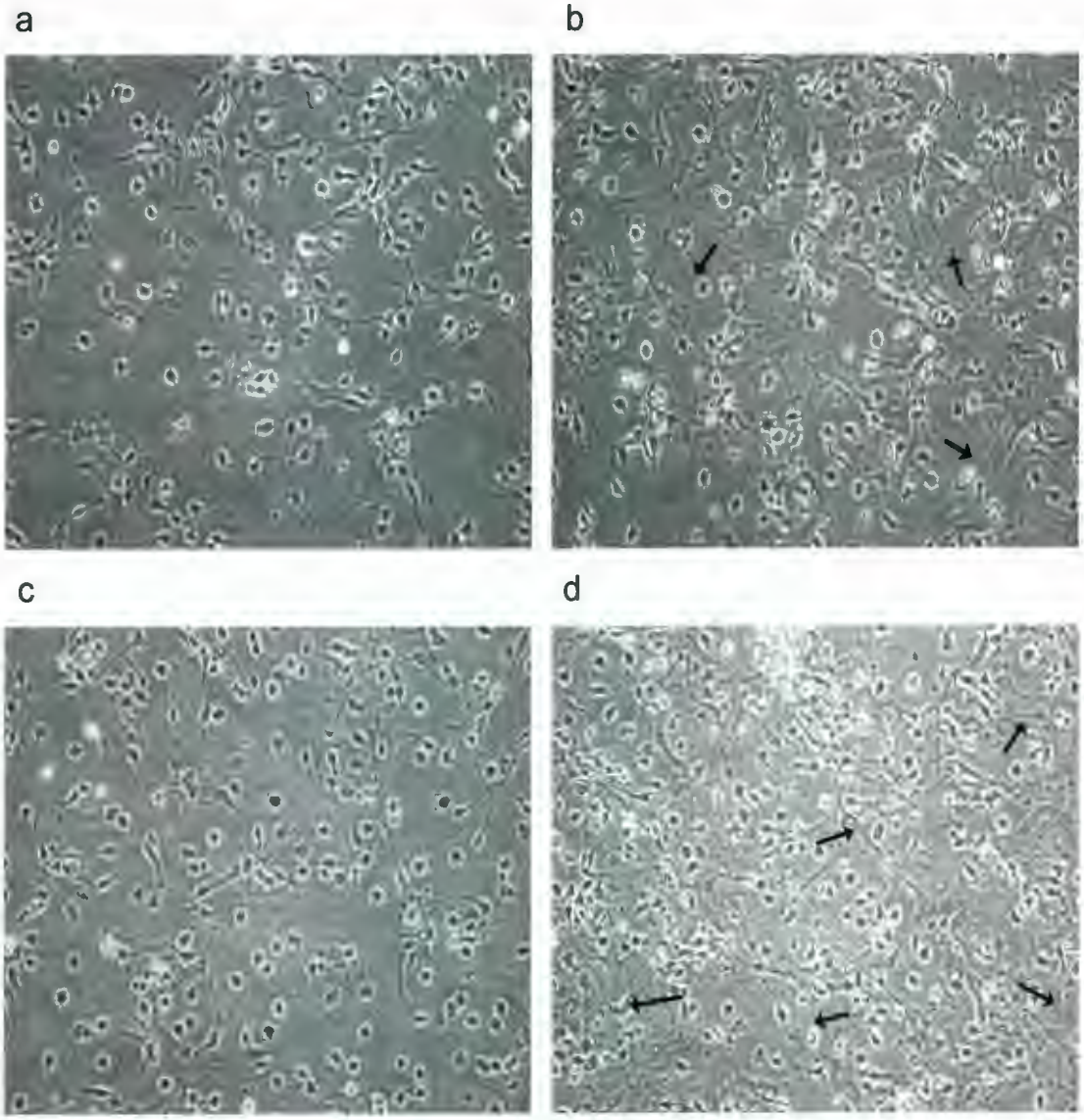
Probed with Hsp27

of $A\beta_{1-42}$ and $A\beta_{25-35}$ on untransfected cells to provide a baseline for future experiments investigating their effect on neurons transfected with Hsp27WT cDNA. Control conditions for $A\beta_{1-42}$ did not receive a peptide, whereas $A\beta_{25-35}$ controls received the reverse control peptide $A\beta_{35-25}$.

3.2.1 $A\beta_{1-42}$ but not $A\beta_{25-35}$ affects untransfected naïve cortical neurons

To investigate the effect of amyloid on cortical neurons, I exposed untransfected cells to Aβ fragments and observed the effect at 24 and 48 hr time points. Earlier time points (4, 6, and 12 hr) were observed but neuronal survival was not affected. It has been suggested that Aβ fibril formation is necessary for neurotoxicity, but recently it has been shown that species that precede fibrilized Aβ are responsible (Kim *et al.*, 2003, Klein, 2002). Further, soluble Aβ was shown to have CNS neurotoxic specificity. Therefore, in my experiments the Aβ peptide was not preincubated for fibril formation prior to addition. Photomicrographs in Fig. 3.4 show the differential effect of Aβ types on morphological characteristics. Those cells exposed to $A\beta_{1-42}$ show clear degeneration, indicated by arrows, whereas cells exposed to $A\beta_{35-25}$ and $A\beta_{25-35}$ remain apparently healthy. To quantify this, a trypan blue exclusion assay was used. Trypan blue dye is excluded from cells with intact membranes but cells that take up the dye are considered to have damaged membranes causing increased cellular permeability. Neurons were photographed using a light microscope and analyzed using ImageJ software. Cells positive for trypan dye were counted relative to total number of cells and survival was measured as percent of control. Results in Fig. 3.5A show that $A\beta_{1-42}$ exposed neurons

Figure 3.4. Phase micrographs provide an example of cortical cultures under different experimental conditions. Panels' a-d show cultures in absence or presence of $A\beta_{1-42}$. a - control at 24 hr, b - $A\beta_{1-42}$ at 24 hr, c - control at 48 hr, d - $A\beta_{1-42}$ at 48 hr. Arrows show clear degeneration in a-d, whereas neurons in e-h treated with $A\beta_{25-35}$ do not. e - control at 24 hr, f - $A\beta_{25-35}$ at 24 hr, g - control at 48 hr, h - $A\beta_{25-35}$ at 48 hr. Degeneration indicated by arrows.


a
b
c
d

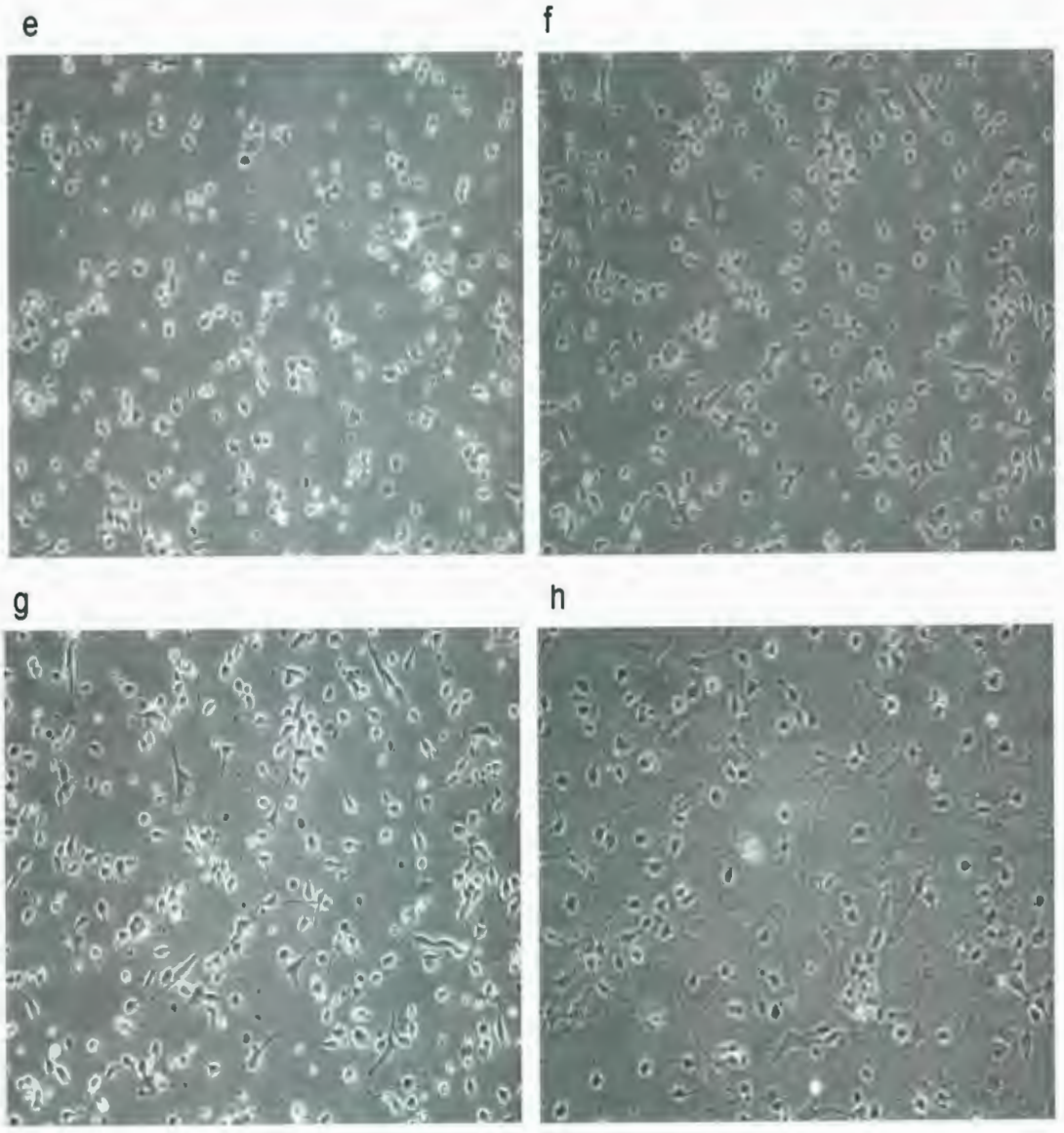
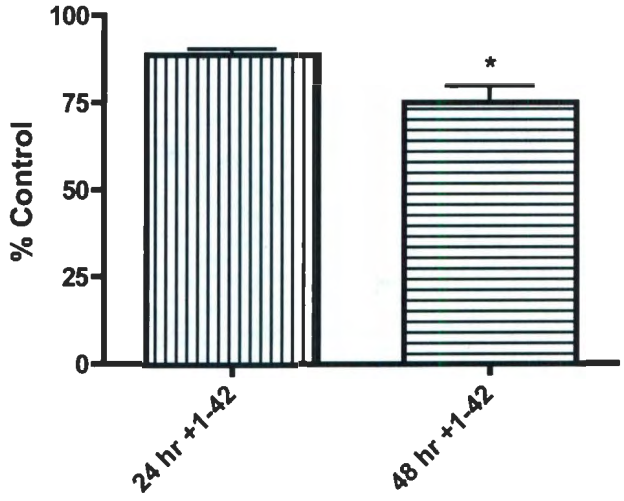
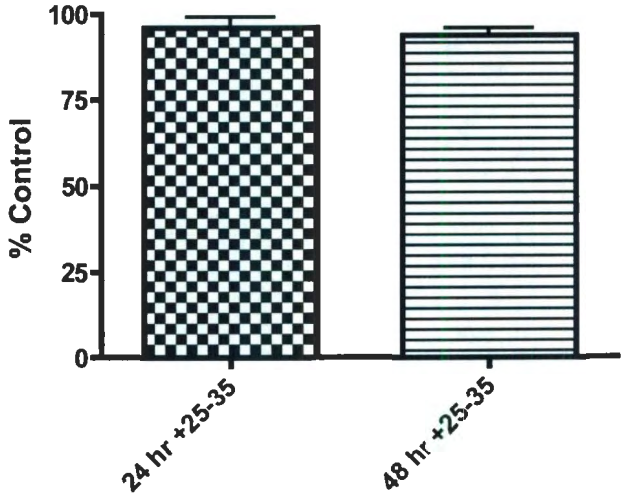

e
f
g
h

Figure 3.5. $A\beta_{1-42}$ but not $A\beta_{25-35}$ kills untransfected neurons. Cell survival was determined using 0.4% trypan blue solution and cells positive for trypan blue were counted in relation to total number of cells per well of a 16 well chamber slide. Values are presented as % of untreated control (control not shown). (A) Shows significant cell death at 48 hr from $A\beta_{1-42}$. However, (B) demonstrates that $A\beta_{25-35}$ does not result in a decrease in survival at 24 and 48 hr time points. Each data point represents several hundred neurons from at least 6 fields of view from multiple experimental replicates (n=3). All data in figure is displayed as mean ± SEM. *$p < 0.05$. (t-test)

A


100
75
50
25
0
% Control
*
24 hr +1-42
48 hr +1-42

B


100
75
50
25
0
% Control
24 hr +25-35
48 hr +25-35

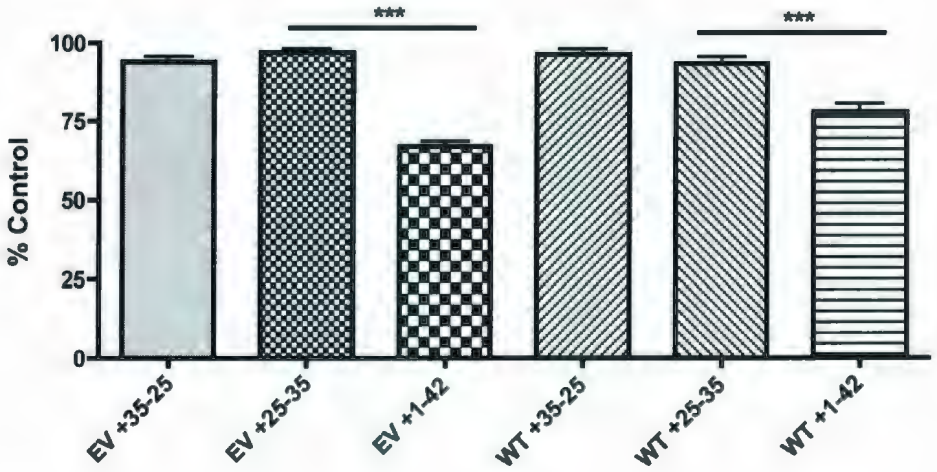
display a significant reduction in survival at 48 hr while $A\beta_{25-35}$ exposed neurons do not (Fig. 3.5B). Previous research (Pike *et al.*, 1993) reported $A\beta_{25-35}$ to be toxic to cortical neurons at a similar concentration and time point but our data indicates otherwise. On the other hand, $A\beta_{1-42}$ has been shown to be toxic to neurons (Evans *et al.*, 2006, Mattson, 2004, Pike *et al.*, 1995, Price *et al.*, 1998) and our results confirm this *in vitro*. Based on these results, I then implemented a similar experimental paradigm on transfected neurons to address specific aim #2, the effect of Hsp27 on survival.

3.3 EV and Hsp27WT transfected neurons treated with amyloid show differential absorbance in the MTS survival assay

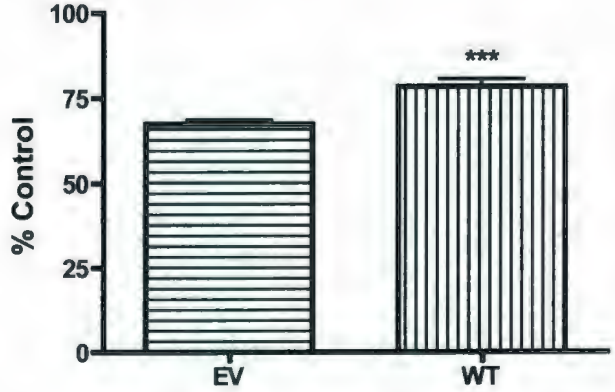
In order to investigate the effect of Hsp27WT cDNA on survival against $A\beta_{1-42}$ and $A\beta_{25-35}$ I used the MTS colorimetric assay. Cells were transfected, plated in 96 well chambers and exposed to $A\beta_{1-42}$, $A\beta_{25-35}$ or its control $A\beta_{35-25}$ peptide for 24 hr. The MTS assay is an indirect assessment of survival where MTS is converted into formazan by mitochondria, producing a color change detectable at 490 nm; decreased absorbance is an indication of decreases in mitochondrial metabolism which has been correlated with decreased cell survival (Mosmann, 1983). Absorbance values for Aβ treated wells were expressed relative to their controls and are displayed in Fig. 3.6 Results indicate that $A\beta_{1-42}$ treated wells demonstrate a significant reduction in absorbance, indicating decreased metabolism confirming our preliminary data in untransfected neurons. More

Figure 3.6. $A\beta_{1-42}$ but not $A\beta_{25-35}$ causes significant reduction in neuronal survival. Neurons were transfected with either EV or Hsp27WT cDNA, plated on poly-D-lysine coated 96 well plates and allowed to recover for 2 days in culture. Cells were exposed to $A\beta_{35-25}$, $A\beta_{25-35}$ or $A\beta_{1-42}$ for 24 hr and then subjected to a MTS colormetric assay measured at 490 nm. Neurons treated with $A\beta_{25-35}$ were also subjected to a trypan blue assay. Absorbance reflects whole well metabolic activity which in turn reflects survival. All conditions are expressed as % of untreated controls (control not shown). A reduction in absorbance is seen for EV and WT transfected cells treated with $A\beta_{1-42}$ but not $A\beta_{25-35}$ or its control peptide $A\beta_{35-25}$. (A). WT transfected neurons demonstrate higher absorbance than EV in $A\beta_{1-42}$ treated wells (B). (C) Shows that $A\beta_{25-35}$ does not result in reduced neuronal survival as determined by trypan blue assay. Cells positive for trypan blue were counted in relation to total number of cells per well of a 16 well chamber slide. Each condition is composed of absorbance readings from seven wells and trypan data is composed of at least 30 neurons, over at least 6 fields of view per condition. All data was composed of multiple experimental replicates (n=3). All data in figure is displayed as mean ± SEM. *** $p < 0.0001$ (ANOVA) and *** $p < 0.0001$ (t-test)

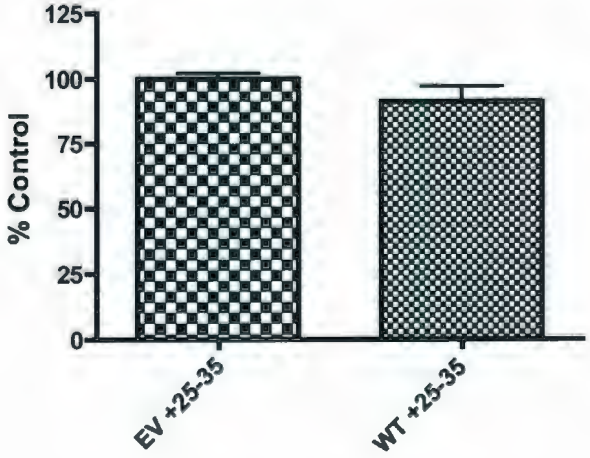
A


***
***
100
75
50
25
0
% Control
EV +35-25
EV +25-35
EV +1-42
WT +35-25
WT +25-35
WT +1-42

B


100
75
50
25
0
% Control
***
EV
WT

C

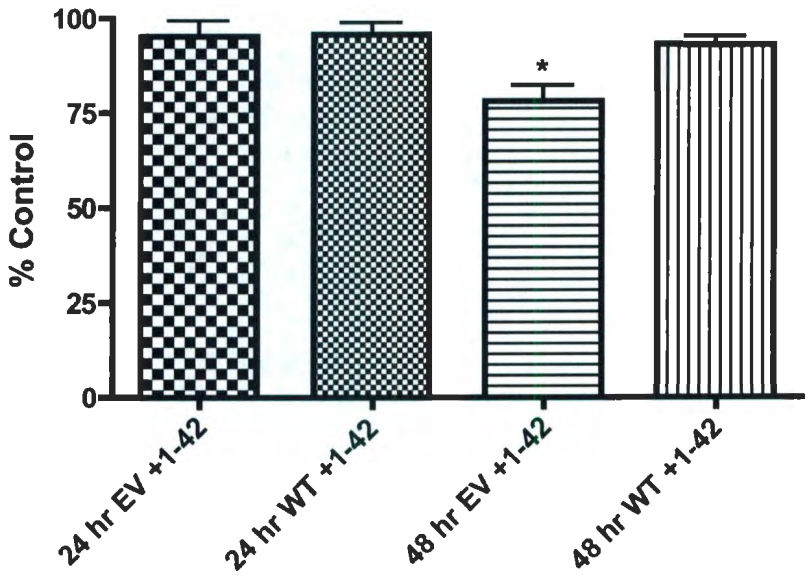

125
100
75
50
25
0
% Control
EV +25-35
WT +25-35

interestingly, it was seen that Hsp27WT cDNA neurons showed greater absorbance than EV transfected neurons (Fig. 3.6B). This may indicate that Hsp27WT cDNA increases neuronal survival. Given that MTS is an indirect measurement of cell survival, I also used the trypan blue exclusion dye assay to more accurately investigate the effects of Aβ types on cell survival at the individual cell level. It can be seen in Fig. 3.6C that $A\beta_{25-35}$ did not have an effect on survival in either the EV or WT condition, reiterating the result found in the previous section that $A\beta_{25-35}$ treatments do not appear to reduce survival, at the concentration used in the time points investigated.

3.3.1 Neurons transfected with Hsp27WT cDNA show greater survival against $A\beta_{1-42}$ than EV transfected neurons.

Given that $A\beta_{1-42}$ but not $A\beta_{25-35}$ was shown to be toxic, I continued to investigate the effects of $A\beta_{1-42}$ but not $A\beta_{25-35}$. In addition, I sought to determine the effects of $A\beta_{1-42}$ treatment on transfected neurons. Cells were plated on 16 well slides and treated with $A\beta_{1-42}$ for 24 and 48 hr. Trypan blue dye was used to determine the effect of Hsp27WT cDNA on survival. Cells positive for Trypan dye were counted relative to total number of transfected cells and survival was measured as percent of control (Fig. 3.7). EV transfected cells show a 17% decline in survival at 48 hr following $A\beta_{1-42}$ treatment, while EGFP-Hsp27WT transfected cells do not (2% decline). This result indicates that transfection of Hsp27WT cDNA has a protective effect after $A\beta_{1-42}$ treatment. To my knowledge this is the first time that transfection of Hsp27WT has been shown to protect cortical neurons against $A\beta_{1-42}$ treatment.

Figure 3.7. Hsp27 cDNA can enhance survival of amyloid-treated neurons. Cells were transfected at the time of plating on poly-D-lysine coated slides and allowed to recover for two days in culture before being exposed to $A\beta_{1-42}$. Cell survival was determined using 0.4% trypan blue solution. Cells positive for trypan blue were counted in relation to total number of cells per well of a 16 well chamber slide. Values are presented as % of untreated controls (control not shown). EV cells shows a significant decrease in survival at 48 hr time point, while WT cells remain at control levels. Data is composed of at least 30 neurons from multiple experimental replicates (n=3). All data in figure is displayed as mean ± SEM. $*p < 0.05$, $***p<0.0001$. (ANOVA)


100
75
50
25
0
% Control
*
24 hr EV +1-42
24 hr WT +1-42
48 hr EV +1-42
48 hr WT +1-42

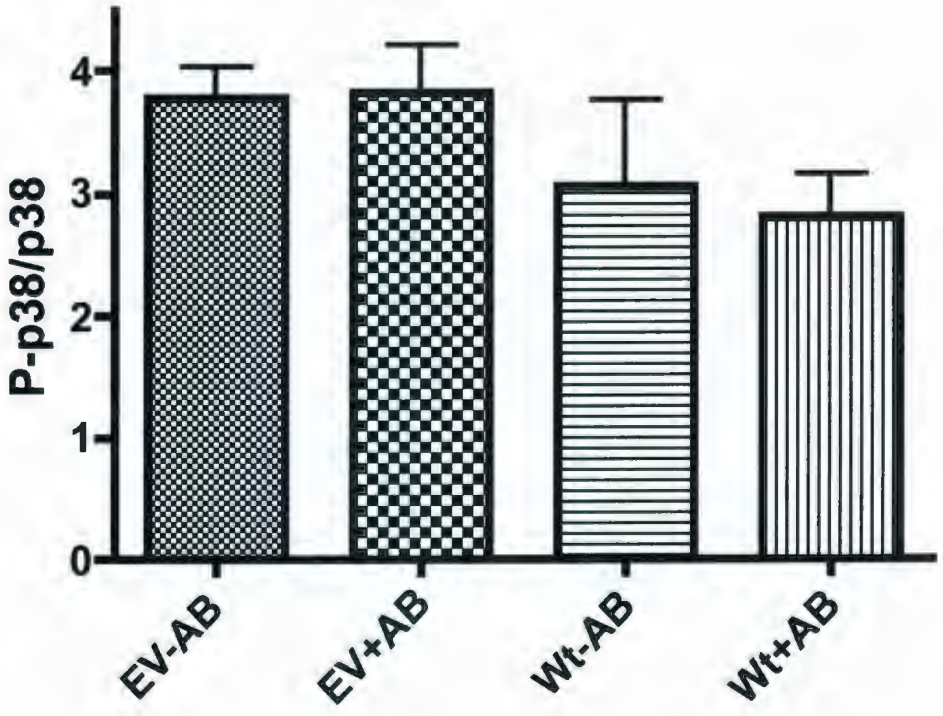
Knowing that Hsp27 has been shown to regulate cell death/survival pathways (Havasi *et al.* 2008, Mearow *et al.*, 2002, Patel *et al.*, 2005), I investigated the effect of $A\beta_{1-42}$ treatment on stress/survival related mechanisms such as activation of p38, Akt, PKCδ, and caspase-3 through Western blot analysis, particularly their activated forms. Western blot and accompanying densitometry in Fig. 3.8 shows a reduction of activated p38 (phospho-p38) in Hsp27WT transfected cells in comparison to those transfected with EV. However, these results were not significant. Unfortunately, my investigation of other signaling proteins is not presented here because it did not reveal a clear response.

Hsp induction has been shown to be specific to stressor type (Taylor and Benjamin, 2005). Consequently, it was possible that $A\beta_{1-42}$ could upregulate endogenous Hsp27. To investigate this possibility, I treated neurons with $A\beta_{1-42}$ for 24 and 48 hr and analyzed protein lysates by Western blot. Data presented in Fig. 3.9 shows that $A\beta_{1-42}$ treatment does not result in increased endogenous Hsp27 expression confirming the previous result that Hsp27 is not expressed in cortical neurons.

3.4 Hsp27WT and Hsp27Δ cDNA increases neurite length at 24 and 48 hr time points.

Previous studies indicate that Hsp27 plays a role in axonal outgrowth and stabilization. Furthermore, Hsp27 has been shown to interact with actin, MAPs (e.g. tau) and intermediate filaments. Knowing this, I decided to examine whether Hsp27 might have a similar effect in cortical neurons. It is possible that Hsp27's protective effects could be a result of axonal growth and stability. To that end, I transfected neurons and

Figure 3.8. p38 is less active in WT transfected cells, as indicated by phosphorylated p38. p38 is part of the stress associated MAP-kinase pathway that leads to the phosphorylation of Hsp27 (Gusev *et al.*, 2002). In my study I sought to investigate p38 activity in response to $A\beta_{1-42}$ treatment. Neurons were transfected at the time of plating with either EV or WT cDNA using Amaxa nucleofector and allowed to recover for 2 days in culture. Cells were exposed to $A\beta_{1-42}$ for 24 hr and collected for Western blot analysis. P-p38 was measured relative to its inactive p38 form and Western blot was subjected to densitometry. Western blot and accompanying densitometry shows that those cultures transfected with Hsp27WT demonstrated less active p38 than those cultures transfected with EV. It should be noted that this result was not significant. Figure represents data from two separate blots and is displayed as mean ± SEM (ANOVA) n=2.


P-p38/p38
4
3
2
1
0
EV-AB
EV+AB
Wt-AB
Wt+AB

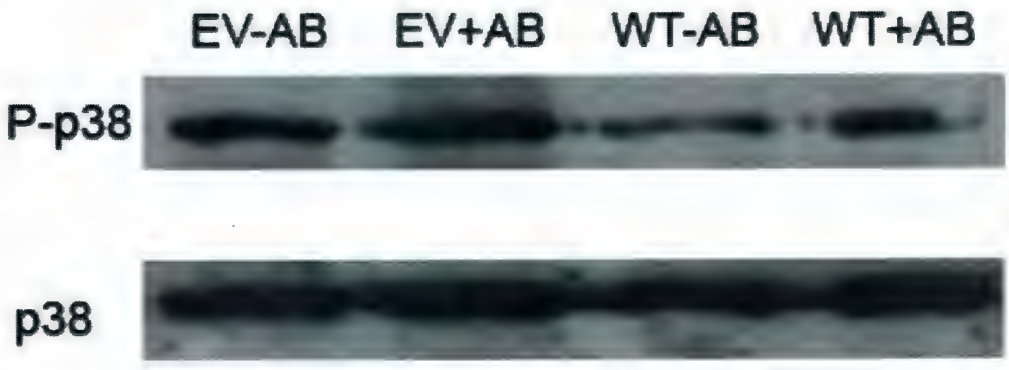
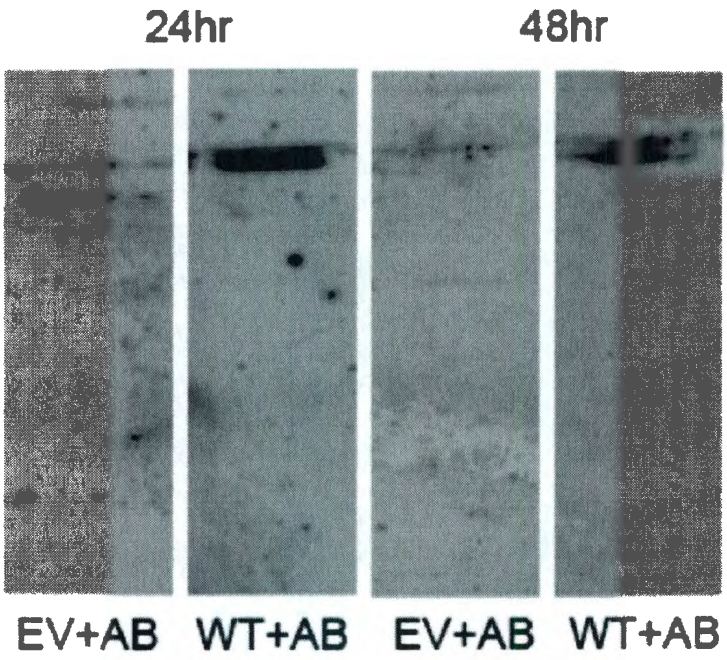

EV-AB
EV+AB
WT-AB
WT+AB
P-p38
p38

Figure 3.9. Western blot shows undetectable levels of endogenous Hsp27 in untransfected and transfected cells treated with $A\beta_{1-42}$. Cortical neurons were plated on poly-D-lysine coated dishes, allowed to mature for two days in culture and then treated with $A\beta_{1-42}$ for 24 and 48 hr. Western blot shows that Hsp27 is not detectable at 24 or 48 hr after $A\beta_{1-42}$ treatment.


24hr
48hr
EGFP-Hsp27
Endogenous Level
EV+AB
WT+AB
EV+AB
WT+AB

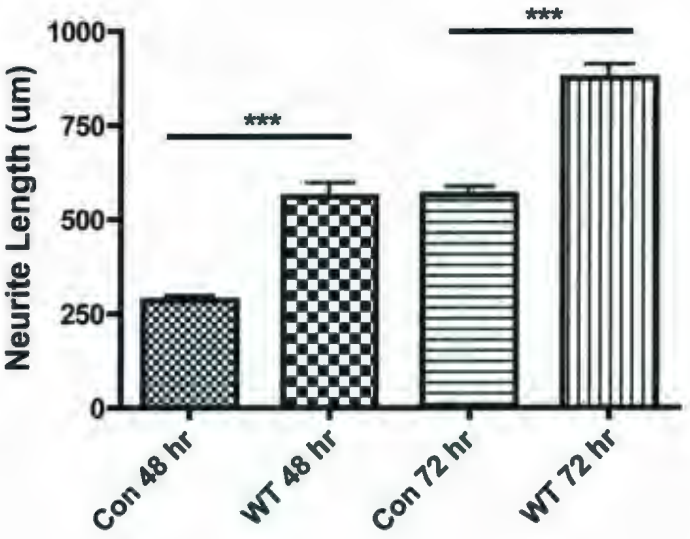
measured the influence of Hsp27 neurite outgrowth using confocal microscopy and ImageJ neuron tracer.

Cells were transfected at the time of plating on 16 well glass slides and fixed at 24 and 48 hr time points. In order to measure cell neurites, cells were immunostained with total neuronal tubulin antibodies and photographed using confocal microscopy. Neurons were photographed using confocal imaging and traced using NeuronJ and total neurite length is displayed in Fig. 3.10. Total neurite length was obtained by tracing all processes from at least 45 separate neurons from each experimental condition and data expressed as the mean from three replicate experiments. Control neurite lengths were measured using untransfected neighboring cells; there was no difference in neurite length between EGFP and untransfected cells (data not shown). Results in Fig. 3.10A indicate that Hsp27WT cDNA resulted in an increase in total neurite length over 48 and 72 hr time points. This indicates that Hsp27WT cDNA increases growth in cortical neurons.

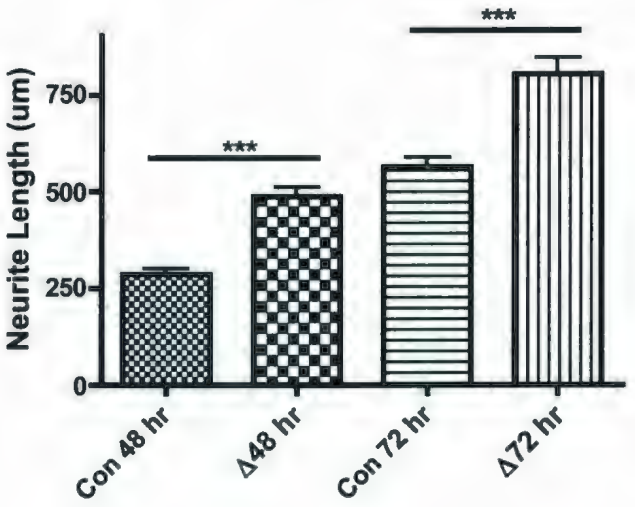
Knowing that phosphorylation affects Hsp27 function in DRG neurons (Williams *et al.*, 2005), I also sought to determine if mutated versions of Hsp27 (i.e. the Δ mutation missing the WDPF domain) had a differential effect on neurite outgrowth. Results in Fig. 3.10B and C show that neurons expressing Hsp27Δ show increased neurite length compared to controls (B), but have similar neurite length as full length Hsp27WT (C). This result implies that the S15 phosphorylation site and surrounding amino acids are not important for increasing neurite length. Representative photos (Fig. 3.10D) illustrate typical neurons in each condition at 48 and 72 hr time points.

Figure 3.10. Wild type Hsp27 results in increased total neurite growth at 48 and 72 hr. Cortical neurons were transfected with empty vector, Hsp27Δ or Hsp27WT cDNA, plated on poly-D-lysine coated slides and immunostained with an antibody directed against neuronal tubulin. Delta-Hsp27 is mutated Hsp27 excluding the WDPF domain and consequently the S15 phosphorylation site . Confocal laser scanning microscopy was used to photograph neurons and NeuronJ, an ImageJ plug-in, was used to trace neurite length. (A) shows that transfection of EGFP-Hsp27WT increases neurite length. Similarly, (B) shows that EGFP-Hsp27Δ transfected neurons significantly increase neurite length but not in comparison to WT conditions (C). Confocal images in (D) provide representative examples of neurons transfected with EV and WT constructs. Images a-c represent typical neurons traced at 48 hr from EV, WT and Δ conditions, respectively, while d-f represent neurons traced at 72 hr. Total neurite length is the sum of all axons and dendritic processes from a single neuron and each data point represents at least 45 neurons from multiple experimental replicates (n=3). All data in figure is displayed as mean ± SEM. ***$p<0.0001$ (ANOVA).

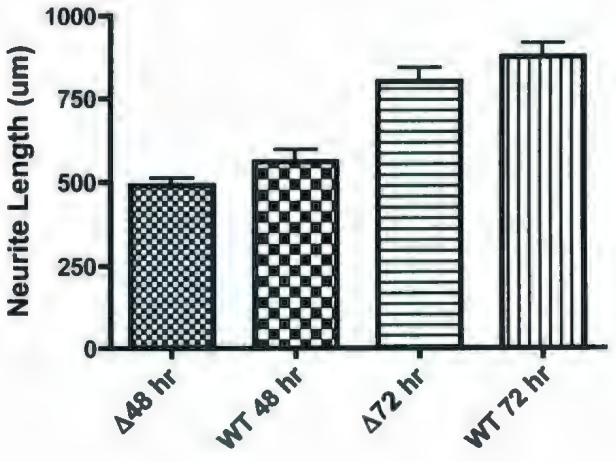
A


Neurite Length (um)
1000
750
500
250
0
***
***
Con 48 hr
WT 48 hr
Con 72 hr
WT 72 hr

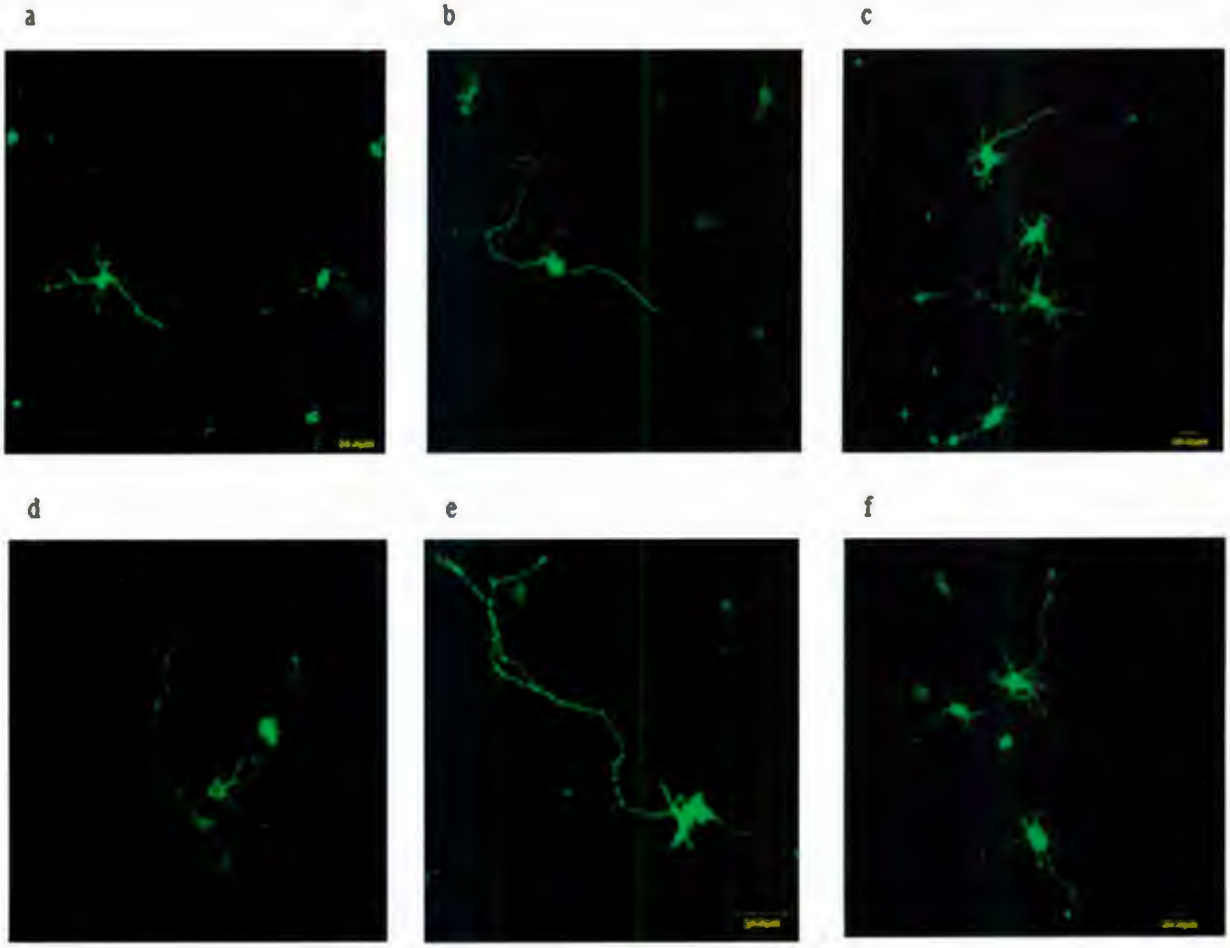
B


Neurite Length (um)
750
500
250
0
***
***
Con 48 hr
Δ48 hr
Con 72 hr
Δ72 hr

C


Neurite Length (um)
1000
750
500
250
0
Δ48 hr
WT 48 hr
Δ72 hr
WT 72 hr

D

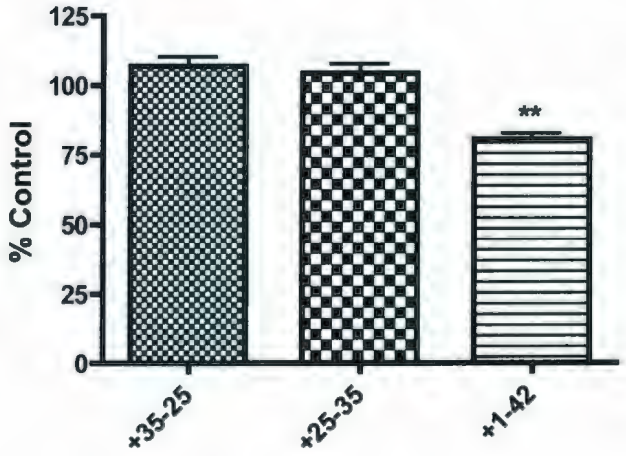


3.4.1 Hsp27Δ mutation shows similar survival effects as Hsp27WT.

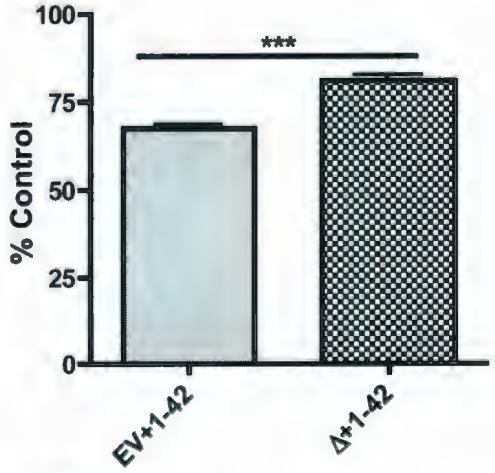
Based on the literature and previous data from our lab (Gusev *et al.*, 2002, Williams *et al.*, 2005), it is known that post translational modification of Hsp27 (i.e. phosphorylation) affects its function. Therefore, in addition to Hsp27Δ effect on neurite length, I sought to investigate the effect of Hsp27Δ on survival. To that end, I transfected and plated neurons on 96 well plates and performed the MTS assay after 24 hr of $A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}42}$ treatment. Results are presented as percent of control in Fig. 3.11. As before, results show that $A\beta_{1\text{-}42}$ but not $A\beta_{25\text{-}35}$ causes a reduction in absorbance, suggesting that $A\beta_{1\text{-}42}$, and not $A\beta_{25\text{-}35}$, is the toxic species (Fig. 3.11A). Fig. 11B shows that cells transfected with Hsp27Δ have greater survival than EV neurons but equal to Hsp27WT (Fig. 3.11C). Hsp27WT and Hsp27Δ constructs do not differentially affect survival according to the MTS assay. This data suggests that Hsp27Δ is as effective at protecting neurons as Hsp27WT. Further analysis using the Trypan assay would contribute to the validity of this result.

Figure 3.11. Hsp27Δ mutation shows similar effects as Hsp27WT. Cells were exposed to $A\beta_{35-25}$, $A\beta_{25-35}$ or $A\beta_{1-42}$ for 24 hr and then subjected to a MTS colormetric assay measured at 490nm. Neurons treated with $A\beta_{1-42}$ were also subjected to a trypan blue assay and were expressed as % of untreated controls (controls not shown). A reduction in absorbance is seen for EV and WT transfected cells treated with $A\beta_{1-42}$ but not $A\beta_{25-35}$ or its control peptide $A\beta_{35-25}$ (A). Trypan data shows that Hsp27Δ results in greater survival than EV neurons (B) but equal to Hsp27WT (C). Each condition is composed of absorbance readings from seven replicate wells and trypan data is composed of at least 30 neurons, over at least 6 fields of view per condition. All data was composed of multiple experimental replicates (n=3). All data in figure is displayed as mean ± SEM. **p<0.001 (ANOVA), *** p<0.0001 (T-test)

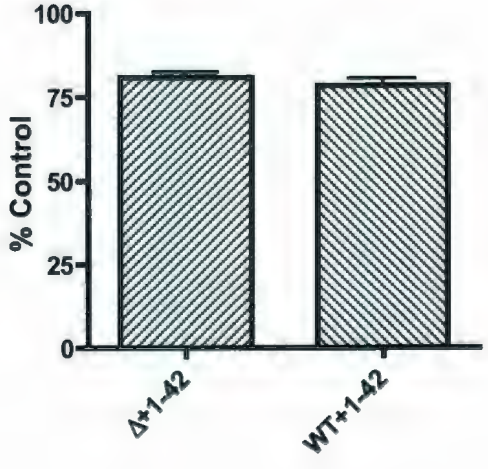
A


125
100
75
50
25
0
% Control
**
+35-25
+25-35
+1-42

B


100
75
50
25
0
% Control
***
EV+1-42
Δ+1-42

C


100
75
50
25
0
% Control
Δ+1-42
WT+1-42

Chapter 4

Discussion

4.1 The Significance of This Study

Hsps can be induced by a variety of environmental stressors such as alcohols, heavy metals and heat stress (Hightower, 1991) and are thought to prevent cell death through various protective mechanisms such as chaperone activity (Becker and Craig, 1994, Evans *et al.*, 2006, Parsell and Lindquist, 1993, Rogalla *et al.*, 1999), cytoskeleton stabilization (Hargis *et al.*, 2004, Hino *et al.*, 2000, Mounier and Arrigo, 2002, Perng *et al.*, 1999), inhibition of apoptotic signals (Garrido *et al.* 1999, Patel *et al.*, 2005) and enhancement of survival pathways (Mearow *et al.*, 2002). Since their discovery in 1962, by Ferruccio Ritossa (1962), Hsps' involvement in the stress response has been studied with intense investigation. However, the role that Hsps play in neurodegenerative diseases such as AD has received relatively little attention. AD is a neurodegenerative disease characterized by premature death of a selective neuronal population, especially within the frontal and temporal lobes (Mattson, 2004), in the presence of NFTs and extracellular neuritic plaques. The amyloid hypothesis asserts that apoptosis is caused by the destabilization of the cytoskeleton in the presence of extracellular amyloid plaques (Mattson, 2004, Price *et al.*, 1998, Song *et al.*, 2002, Tanzi and Bertram, 2001). My thesis sought to investigate the putative protective role of Hsp27 in cortical neurons *in vitro* after treatment with various types of Aβ, namely $A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}42}$. Firstly, I investigated the endogenous expression of Hsp27, secondly, the protective role of Hsp27 against Aβ treatment, and thirdly, the role that Hsp27 has in neurite outgrowth with a

particular investigation of mutated Hsp27Δ (Hsp27Δ lacking the WDPF domain and S15 phosphorylation site), knowing that phosphorylation can influence Hsp27 function (Gusev *et al.*, 2002, Lelj-Garolla and Mauk, 2005, Takai *et al.*, 2007, Williams *et al.*, 2005).

4.2 Cortical Neurons Lack Endogenous Hsp27

Hsps are conservative proteins whose expression varies between tissue and cell type. For example, Bechtold and Brown (2003) found that hyperthermic induced expression of Hsp27 in the cortex was restricted to astrocytes. On the other hand, Hsp70 is expressed at low levels in the brain under normal conditions but is highly inducible in response to injury such as hyperthermia, ischemia or seizure (Armstrong *et al.* 1996, Krueger *et al.* 1999, Manzerra *et al.*, 1997). Based on the literature and a number of studies in our lab (Mearow *et al.*, 2002, Williams *et al.*, 2005), it has been shown that, in certain *in vitro* systems, namely PC12 and cultured DRG neurons, Hsp27 expression can be induced by sub-lethal heat shock to afford protection or tolerance against a subsequent more noxious stimulus.

In order to investigate Hsp27's role in the protection of cortical neurons against Aβ, it seemed appropriate to first investigate endogenous Hsp27 expression. My study shows that Hsp27 is not present in cortical neurons, endogenously at 24 or 48 hr after heat shock. Since some sHsps require a chemical, and not heat, stress for their upregulation, we also analyzed protein lysates from cells exposed to Aβ peptides and did not find any evidence of Hsp27 expression. Furthermore, we tested for Hsp27 expression

at 3, 6, and 12 hr time points post heat shock but did not find any indication that Hsp27 is expressed endogenously. In agreement with previous aforementioned studies, my preliminary experiments show that Hsp27 is not present in cortical neurons *in vitro*. It should be noted that we also conducted PCR analysis on cortical cultures for Hsp27 and heat shock factor-1 (Hsf-1, the factor responsible for Hsp27 upregulation). It was found that while both Hsp27 and Hsf1 mRNA were present they did not display any increases after heat shock (Nafar and Mearow, unpublished data). During my preliminary experiments, I noticed that heat shock cultures appeared unhealthy (e.g. degenerative processes and fragmented cell bodies) following heat shock at 24 and 48 hr time points and investigated the survival of cortical neurons by trypan blue assay. It was seen that heat shock exposure resulted in significantly less survival in comparison to control at 24 and 48 hr time points as assessed by the trypan survival assay. Given the delicate nature and stress associated with culturing primary neurons it is possible that neurons may not have been able to withstand a heat shock treatment of 45°C for 15 min. That being said, increasing the recovery period after plating may allow neurons to more fully recover and result in healthier, more durable neurons capable of surviving heat shock treatment. Furthermore, previous studies (Mearow *et al.*, 2002) performed a media change 1 hr prior to heat shock whereas my experiments did not. A media change may have offered a protective effect due to the fresh addition of antioxidants, glucose and vitamins. Perhaps a media change would have allowed for greater survival in heat shock treated cultures.

4.3 Differential Toxicity of $A\beta_{1-42}$ and $A\beta_{25-25}$

Exposure of cells to Aβ in culture is a well established AD model. Aβ peptides of various lengths have been shown to be toxic in culture (Hardy and Selkoe, 2002), but some studies have shown $A\beta_{1-42}$ and $A\beta_{25-35}$ to be the predominant toxic species (Kawahara and Kuroda, 2000, Pike *et al.*, 1993, Resende *et al.*, 2007). $A\beta_{1-42}$ has been shown to have a high susceptibility to fibrilize, self aggregate (Harper *et al.*, 1999, Pike *et al.*, 1995), generate hydrogen peroxide species (Mattson, 2004), deposit early and disproportionately contribute to neuronal toxicity (Price *et al.*, 1998). On the other hand, $A\beta_{25-35}$ has been shown to be the biologically toxic fragment of the full length Aβ peptide (Yankner *et al.*, 1990). Similarly, both $A\beta_{1-42}$ and $A\beta_{25-35}$ have been attributed to forming pores in the cellular membrane allowing for Ca^{++} influx and subsequent activation of apoptosis (Kawahara and Kuroda, 2000, Resende *et al.*, 2007). Both are capable of forming fibrilized aggregates but some have suggested that Aβ fibril formation is not necessary for neurotoxicity (Kim *et al.*, 2003, Klein, 2002, Walsh and Selkoe 2004). Consequently, I treated cortical neurons with fresh Aβ (i.e. not pre-incubated) and assessed baseline effects using photomicrographs and the trypan blue assay. However, it is possible that Aβ may begin to aggregate in culture as it has been shown that Aβ aggregation is temperature and time dependent. Typically, Aβ shows drastic aggregation after 7 days *in vitro* (Pike *et al.*, 1995) but it is possible that Aβ could aggregate after two days in culture.

Contrary to the literature, my observations show that $A\beta_{25-35}$ did not result in significant death in cortical neurons *in vitro*, while on the other hand $A\beta_{1-42}$ caused a

significant reduction in survival. The observation that $A\beta_{1-42}$, but not $A\beta_{25-35}$, resulted in significant cell death might be due to the following. Neurotoxicity is directly related to aggregation state and $A\beta_{1-42}$ has a high susceptibility to aggregate (Harper *et al.*, 1999). Pike *et al.* (1995) suggested that peptide hydrophobicity may play a role in aggregation, as the amount of hydrophobic content in each peptide was proportional to its toxicity. Considering that $A\beta_{1-42}$ contains twice as many transmembrane (hydrophobic) amino acids as $A\beta_{25-35}$ (Fig. 4.1), $A\beta_{1-42}$ may have aggregated sooner than $A\beta_{25-35}$, resulting in earlier toxicity. Perhaps if I lengthened the duration of Aβ exposure, allowing for $A\beta_{25-35}$ to aggregate, I would observe comparable cell death. Nonetheless, it seems that $A\beta_{1-42}$ is more toxic at earlier time points.

$A\beta_{25-35}$ has previously been shown to be toxic at 25 μM but our studies did not confirm this result (Kawahara and Kuroda, 2000, Pike *et al.*, 1993, Resende *et al.*, 2007). I demonstrated that $A\beta_{25-35}$ treatment does not result in toxicity as determined by the MTS and trypan blue survival assay. A possible reason for this discrepancy is that previous *in vitro* studies showing toxicity after 25 μM Aβ treatment did not use media supplemented with antioxidants (Pike *et al.*, 1995), whereas my culture media contained antioxidants from B27 supplement. Given that Aβ has been shown to generate oxidative species (Mattson, 2004), it is possible that antioxidants included in B27 had a protective effect against Aβ generated oxidative species. Antioxidant free B27 is available and it might be useful to replicate the same experiments described here with antioxidant-free B27 supplement.

4.4 Hsp27 cDNA Protects Neurons After $A\beta_{1-42}$ Treatment

Hsp's actions to prevent cell death have been studied in great detail and the mechanisms by which it has been suggested to act include the following: (1) inhibition of apoptosis and/or upregulation of survival pathways, (2) chaperone activity, and (3) stabilization of the cytoskeleton. There may be other mechanisms by which Hsp27 acts to protect cells but these are the main processes of concern in my study. It was shown that Hsp27 cDNA protected neurons against $A\beta_{1-42}$ treatment to some degree. In the following sections, 4.4.1-4.4.3, I will consider each protective property in relation to my observations.

4.4.1 Inhibition of Apoptosis and Enhancement of Survival Pathways

Hsp27 has been shown to inhibit cell death by inhibiting apoptosis and promoting survival. For example, Hsp27 has been shown to prevent cell death in cells over-expressing Hsp27 by attenuating caspase-3 and -9 activation (Garrido *et al.*, 1999, Mearow *et al.*, 2002). In addition, Hsp27 has been shown to protect cells by promoting Akt activity and it appeared that Hsp27 interacted with Akt in order to sustain its activity, suggesting that Hsp27 interacts with survival proteins in order to protect neurons (Mearow *et al.*, 2002). Recently, Hsp27 has been shown to regulate Akt via PI3-K and concomitantly inhibit Bax mediated cell death (Havasi *et al.*, 2008). Moreover, Hsp27 can enhance survival through cytochrome C and fas pathways (Patel *et al.*, 2005), showing that Hsp27's anti-apoptotic/survival effects are ubiquitous in cell death/survival pathways.

Knowing this I investigated several stress, apoptotic, and survival signals through Western blot analysis, particularly their activated forms.

pAkt Phosphorylated Akt is an indication of activated Akt and is an integral component of survival signaling in many cell types, including neurons (Bijur and Jope, 2000, Konishi *et al.*, 1997, Mearow *et al.* 2002). I sought to explain Hsp27's protective effect in part through Akt signaling but unfortunately, I was unable to find a clear response. A possible reason for why I was unable to find activation of Akt is that Akt phosphorylation is dynamic and can change in a matter of minutes. Some studies in our lab have shown peak phosphorylation at 3 hr post heat shock (Mearow *et al.*, 2002). In hindsight, analysis of Akt at shorter time points would have been appropriate, and could have allowed me to determine whether any survival effects were due to activation of this signaling pathway.

P-p38 p38 is part of the stress associated MAP-kinase pathway that leads to the phosphorylation of Hsp27 (Gusev *et al.*, 2002). Phosphorylated p38 (P-p38) acts on MAPKAP-K2 to phosphorylate Hsp27 (Gusev *et al.*, 2002) and our lab has shown its activation as early as 6 hr, and up to 24 hr post heat stress (Mearow *et al.*, 2002). In my study I sought to investigate p38 activity in response to Aβ treatment and although this data is not significant, it was seen that those cultures transfected with Hsp27WT demonstrated a trend that p38 was less active than those cultures transfected with empty vector. This suggests that Hsp27WT is playing a role to reduce stress in cultures treated with $A\beta_{1-42}$. Considering the literature on Hsp27's involvement in stress (Fig. 4.2) and

apoptotic pathways, I also investigated PKCδ, JNK and Caspase - 3 but results were not consistent. This was likely due to the low amount of protein collected per condition. It was often the case that I would collect 30 μg of protein or less per condition and in some of these experiments I did not have equal amounts of sample between lanes. Ideally, I would have needed to collect more than 50 μg of protein per sample and load equivalent amounts on my gels. Furthermore, these signals are often present at very low levels. It is also possible that the time points I was analyzing were not optimal to observe alterations in the activation of these proteins.

4.4.2 Hsps as Molecular Chaperones

It is possible that Hsp27 promoted survival by acting as a molecular chaperone, as it has been shown that Hsp27 can refold stress-denatured proteins and shuttle damaged proteins to the degradation pathway (Becker and Craig, 1994, Parsell and Lindquist, 1993, Rogalla *et al.*, 1999), two especially important processes in an AD model considering that Aβ treatment has been shown to act in a hierarchical manner to produce NFTs (aggregates of protein tau), resulting in a clogging of the axon and eventual cell death. I have shown that the presence of Hsp27WT can protect neurons from Aβ induced toxicity and it is reasonable to suggest that Hsp27 may have acted to clear aggregated tau as Hsp27 has been shown to promote cell survival by binding hyperphosphorylated tau, leading to its dephosphorylation and degradation (Shimura *et al.*, 2004). Clearing of NFTs from the axon would allow for normal axonal transport and cytoskeleton growth.

An alternative explanation is that Hsp27 could have acted as an antioxidant, minimizing the effects of oxidative species generated by $A\beta_{1-42}$ (Mattson, 2004). For example, Hsp27 has been shown to protect neuronal cells against superoxide dismutase mutant-induced cell death (Patel *et al.*, 2005), implying that Hsp27 can act as an antioxidant to combat the negative metabolic effects of $A\beta_{1-42}$. This idea is only speculative and a variety of biochemical experiments would be required to make this conclusion.

4.4.3 Hsps Act to Stabilize the Cytoskeleton and Increase Neurite Outgrowth

It is possible that Hsp27's protective ability may be independent of signaling pathways or chaperone activity. For example, Hsp27 has been shown to interact directly with the cytoskeleton (Huot *et al.*, 1996, Lavoie *et al.*, 1993, Miron *et al.*, 1991) and some have inferred that it can stabilize via actin capping (Benndorf *et al.* 1994, Mounier and Arrigo, 2002). Hsp27 has been shown to bind cytoskeletal related proteins, such as vimentin, to maintain a soluble pool of subunits that maintain network integrity (Lee *et al.*, 2005), while the related αβ-crystallin has demonstrated a similar effect in maintaining GFAP networks (Perng *et al.*, 2004), suggesting a general role of Hsp27 involvement in neuro- and intermediate filament network stability. Furthermore, Williams *et al.* (2006) demonstrated that Hsp27 is required for normal neurite outgrowth by showing that the amount of Hsp27 in DRG neurons correlated to neurite length and complexity. Interestingly, my study shows that Hsp27 both protects against $A\beta_{1-42}$ treatment and increases neurite length.

I suggest that Hsp27 is stabilizing the axon, resulting in both a prevention of destabilization under stress (i.e. Aβ treatment) and increased growth under normal conditions. Hsp27 can interact with microtubules in muscle cells and some cell lines (Hargis *et al.*, 2004, Hino *et al.*, 2000) and has been inferred not only to stabilize actin, but neurofilaments, intermediate filaments, MTs and tubulin. This suggests that the addition of Hsp27 cDNA could result in a healthier, more stable cytoskeleton with more vigorous growth, thus explaining the effect of Hsp27 on neurons in my current study. Unfortunately, definitively proving this would require a great deal of analysis beyond my project and I can only speculate the mechanism by which Hsp27 could modulate the cytoskeleton. Nonetheless, this provides an alternative line of research from which I could investigate the effect of Hsp27 on other regions in the CNS. Here, I have suggested that Hsp27 can promote survival and stabilize the axonal cytoskeleton. Given that the projections between the EC, hippocampus and cortex degenerate early in AD (Mattson and Magnus, 2006) it would be reasonable to investigate the effect of Hsp27 on axon stability in the entorhinal cortex and limbic structures. Previous studies have examined neurite length in EC neurons (Tomozawa and Appel, 1986, Ohshima et al., 2008) and a similar study investigating the effect of Hsp27 on neurite stability with $A\beta_{1-42}$ treatments *in vitro* in EC neurons may provide a partial explanation for early degeneration in these areas.

My results support the hypothesis that Hsp27 can promote survival in the face of amyloid treatment. My results provide preliminary data to support further study on the

role of Hsp27 in the protection of cytoskeletal elements as well as investigating relevant signaling pathways.

4.5 The Effect of Hsp27Δ on Neurite Outgrowth and Survival

In order to investigate the role of phosphorylation on neurite outgrowth, I employed Hsp27Δ cDNA, a mutated version of Hsp27 lacking the WDPF domain. Although there was no significant difference between Hsp27Δ and Hsp27WT, it is interesting that Hsp27Δ neurons had shorter processes than Hsp27WT at both time points. My results show a trend that neurons without the WDPF and consequently no phosphorylation on the S15 site had shorter neuritic processes. Previous literature suggest that non-phosphorylated Hsp27 binds and stabilizes actin filaments by binding to the fast growing plus end, acting as an actin capping protein, inhibiting polymerization (Benndorf *et al.*, 1994, Miron *et al.*, 1991). Our lab has shown that an inhibition of phosphorylation at the S15 site by p38 MAPK inhibitor resulted in reduced neurite growth or proper neurite extension (Williams *et al.*, 2006). Here, I have shown that Hsp27Δ neurons show increased neurite length in comparison to control transfected cells but not to that of Hsp27WT neurons.

This result led me to ask whether Hsp27Δ could have an effect on survival after Aβ treatment. It has been shown that the WDPF domain plays a significant role in oligomeric structure and chaperone activity (Gusev *et al.*, 2002). With that in mind, I returned to a previous experimental design and exposed Hsp27Δ transfected neurons to both $A\beta_{25-35}$ and $A\beta_{1-42}$ for 24 hr. Cell survival assays showed there was little difference

between the Hsp27Δ and Hsp27WT in terms of promoting survival after $A\beta_{1-42}$ treatment. This suggests that Hsp27Δ is as effective as Hsp27WT at protecting neurons after $A\beta_{1-42}$. These results provide an insight into future directions on the effect of phosphorylation of Hsp27 in survival against $A\beta_{1-42}$ treatment. Further analysis using the trypan blue assay would confirm the effect of Hsp27Δ on survival in $A\beta_{1-42}$ treated neurons.

4.6 Future Directions

This study has investigated the role of Hsp27 in cortical neurons with respect to its effects on neurite outgrowth and survival. It has addressed the hypothesis that Hsp27 could promote survival and stabilize the axonal cytoskeleton resulting in maintenance of neurite growth and axonal transport in the face of stressors such as Aβ. It was shown that Hsp27 is both important for survival and neurite outgrowth. However, a number of questions remain concerning the mechanisms that attenuate $A\beta_{1-42}$ induced neuronal death in Hsp27 transfected cortical neurons. For instance, it is unknown if Hsp27 is protecting neurons by acting to reduce cell death via signaling pathways or chaperone activity, or both. In addition, it remains unknown how Hsp27 facilitates neurite growth and whether its survival effects are linked by stabilizing the cytoskeleton.

Given AD's impact on the EC and basal forebrain (Davies and Maloney, 1976, Cassel *et al.*, 2008, Frolich, 2002, Muth *et al.*, 2009 Pappas *et al.*, 2000), it would be relevant to investigate the protective role of Hsp27 in these neurons. An analysis of Hsp27's role in protecting entorhinal neurons *in vitro* against $A\beta_{1-42}$ has yet to be conducted and such a study would provide a more comprehensive investigation of

Hsp27's role in neuroprotection of the CNS. Hsp27 has been shown to be upregulated in glial (Bidmon *et al.*, 2005) and neuronal cells (Kato *et al.*, 1991) of the EC and the limbic system in response to ischemia and seizure, respectively. More interestingly, it was shown that HspA5 (Grp78, glucose regulatory protein) and Hsp72 were upregulated in surviving neurons of the EC and hippocampus of AD patients, suggesting that Hsps can play a role in cell survival in these regions (Hamos *et al.*, 1991, Ohshima *et al.*, 2008). Thus, it seems reasonable to further investigate the effect of $A\beta_{1-42}$ on Hsp27 transfected EC neurons *in vitro*. I should note that my initial experiments employed the use of cultured hippocampal neurons but because of the high number of non-neuronal cells in these cultures, I decided to pursue my experiments with cortical cultures, where there was less glial cell contamination.

I demonstrated that $A\beta_{25-35}$ was not toxic to cortical neurons *in vitro*, while previous studies at the same concentration have concluded that $A\beta_{25-35}$ is the toxic fragment of the full length peptide (Pike *et al.*, 1993) and reasons for this discrepancy are unknown. Given the literature and Hsp27's effect on survival and neurite growth in my study, it is my suggestion that Hsp27 acts to stabilize the axon during stress and prevents its degradation and neuronal death. However, many more experiments are required to support this conclusion and given the scope of this topic, my thesis can provide a basis which to further investigate this conjecture.

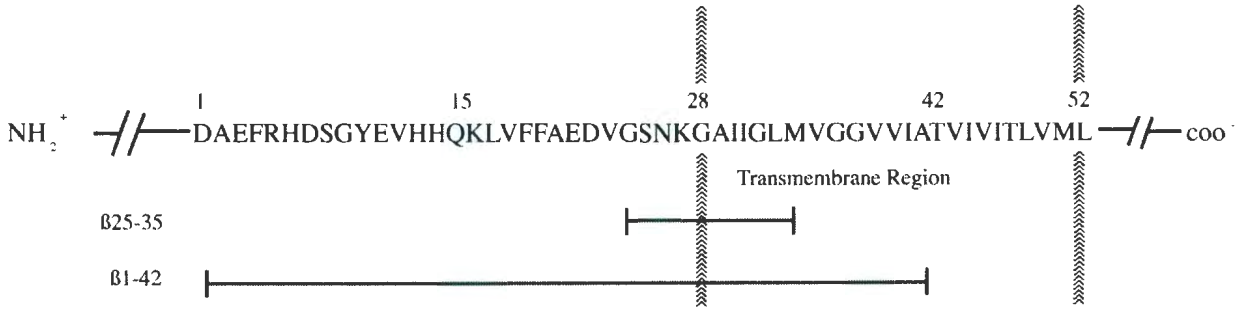


Figure 4.1. Hydrophobic regions in $A\beta_{1-42}$ and $A\beta_{25-35}$. It is thought that hydrophobicity is related to toxicity (Adapted from Pike *et al.*, 1993).

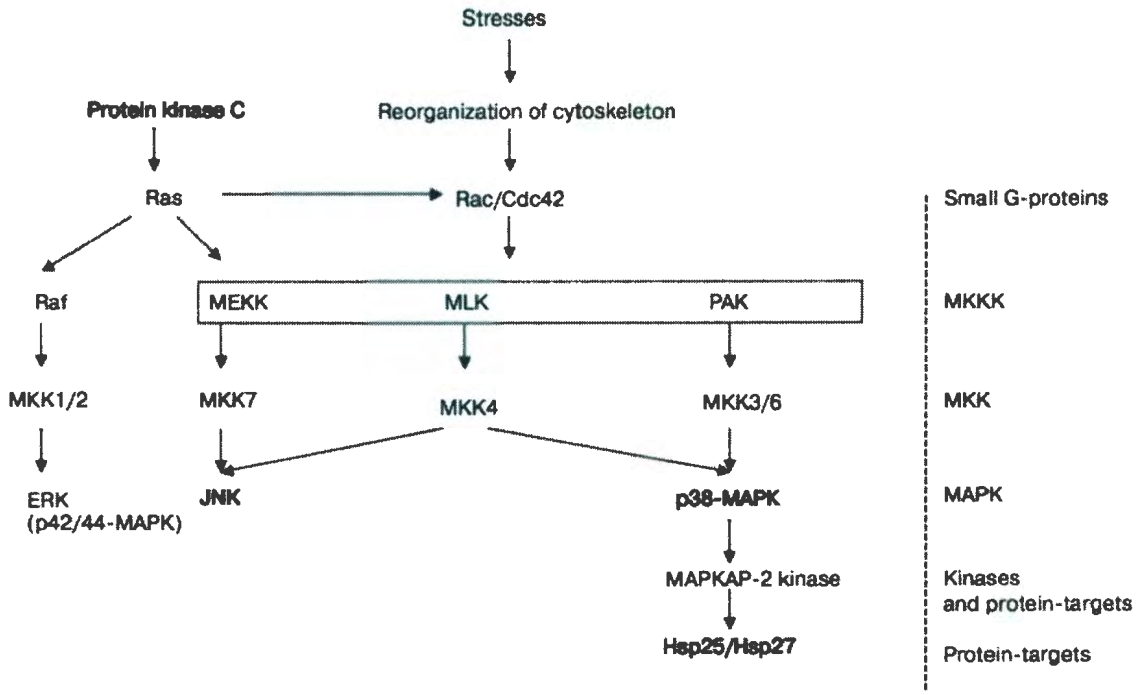


Figure 4.2. Stress response involving several signaling pathways, including the phosphorylation of Hsp27 (Adapted from Gusev *et al.*, 2002).

References

Ackerley S, James PA, Kalli A, French S, Davies KE, Talbot K (2006) A mutation in the small heat-shock protein HSPB1 leading to distal hereditary motor neuronopathy disrupts neurofilament assembly and the axonal transport of specific cellular cargoes. Hum Mol Genet 15:347-354.

Akbar MT, Lundberg AM, Liu K, Vidyadaran S, Wells KE, Dolatshad H, Wynn S, Wells DJ, Latchman DS, de Belleroche J (2003) The neuroprotective effects of heat shock protein 27 overexpression in transgenic animals against kainate-induced seizures and hippocampal cell death. J Biol Chem 278:19956-19965.

Alafuzoff I, Pikkarainen M, Arzberger T, Thal DR, Al-Sarraj S, Bell J, Bodi I, Budka H, Capetillo-Zarate E, Ferrer I, Gelpi E, Gentleman S, Giaccone G, Kavantzas N, King A, Korkolopoulou P, Kovacs GG, Meyronet D, Monoranu C, Parchi P, Patsouris E, Roggendorf W, Stadelmann C, Streichenberger N, Tagliavini F, Kretzschmar H (2008) Inter-laboratory comparison of neuropathological assessments of beta-amyloid protein: a study of the BrainNet Europe consortium. Acta Neuropathol 115:533-546.

Amin V, Cumming DV, Coffin RS, Latchman DS (1995) The degree of protection provided to neuronal cells by a pre-conditioning stress correlates with the amount of heat shock protein 70 it induces and not with the similarity of the subsequent stress. Neurosci Lett 200:85-88.

Armstrong JN, Plumier JC, Robertson HA, Currie RW (1996) The inducible 70,000 molecular/weight heat shock protein is expressed in the degenerating dentate hilus and piriform cortex after systemic administration of kainic acid in the rat. Neuroscience 74:685-693.

Arrigo AP, Simon S, Gibert B, Kretz-Remy C, Nivon M, Czekalla A, Guillet D, Moulin M, Diaz-Latoud C, Vicart P (2007) Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets. FEBS Lett 581:3665-3674.

Bales KR, Verina T, Dodel RC, Du Y, Altstiel L, Bender M, Hyslop P, Johnstone EM, Little SP, Cummins DJ, Piccardo P, Ghetti B, Paul SM (1997) Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition. Nat Genet 17:263-264.

Bechtold DA, Brown IR (2003) Induction of Hsp27 and Hsp32 stress proteins and vimentin in glial cells of the rat hippocampus following hyperthermia. Neurochem Res 28:1163-1173.

Becker J, Craig EA (1994) Heat-shock proteins as molecular chaperones. Eur J Biochem 219:11-23.

Benndorf R, Hayess K, Ryazantsev S, Wieske M, Behlke J, Lutsch G (1994) Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. J Biol Chem 269:20780-20784.

Bidmon HJ, Gorg B, Palomero-Gallagher N, Schliess F, Gorji A, Speckmann EJ, Zilles K (2005) Bilateral, vascular and perivascular glial upregulation of heat shock protein-27 after repeated epileptic seizures. J Chem Neuroanat 30:1-16.

Bierer LM, Haroutunian V, Gabriel S, Knott PJ, Carlin LS, Purohit DP, Perl DP, Schmeidler J, Kanof P, Davis KL (1995) Neurochemical correlates of dementia severity in Alzheimer's disease: relative importance of the cholinergic deficits. J Neurochem 64:749-760.

Bijur GN, Jope RS (2000) Opposing actions of phosphatidylinositol 3-kinase and glycogen synthase kinase-3beta in the regulation of HSF-1 activity. J Neurochem 75:2401-2408.

Blass JP (2001) Brain metabolism and brain disease: is metabolic deficiency the proximate cause of Alzheimer dementia? J Neurosci Res 66:851-856.

Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del Tredici K (2006) Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol 112:389-404.

Busciglio J, Lorenzo A, Yeh J, Yankner BA (1995) beta-amyloid fibrils induce tau phosphorylation and loss of microtubule binding. Neuron 14:879-888.

Cassel JC, Mathis C, Majchrzak M, Moreau PH, Dalrymple-Alford JC (2008) Coexisting cholinergic and parahippocampal degeneration: a key to memory loss in dementia and a challenge for transgenic models? Neurodegener Dis 5:304-317.

Chapman PF, White GL, Jones MW, Cooper-Blacketer D, Marshall VJ, Irizarry M, Younkin L, Good MA, Bliss TV, Hyman BT, Younkin SG, Hsiao KK (1999) Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. Nat Neurosci 2:271-276.

Costantini C, Della-Bianca V, Formaggio E, Chiamulera C, Montresor A, Rossi F (2005) The expression of p75 neurotrophin receptor protects against the neurotoxicity of soluble oligomers of beta-amyloid. Exp Cell Res 311:126-134.

Cotman CW, Anderson AJ (2000) The brain's microenvironment, early functional loss, and the conversion to Alzheimer's disease. Ann N Y Acad Sci 924:112-116.

Cottrell BA, Galvan V, Banwait S, Gorostiza O, Lombardo CR, Williams T, Schilling B, Peel A, Gibson B, Koo EH, Link CD, Bredesen DE (2005) A pilot proteomic study of amyloid precursor interactors in Alzheimer's disease. Ann Neurol 58:277-289.

D'Andrea MR, Nagele RG, Wang HY, Peterson PA, Lee DH (2001) Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology 38:120-134.

Davies CA, Mann DM, Sumpter PQ, Yates PO (1987) A quantitative morphometric analysis of the neuronal and synaptic content of the frontal and temporal cortex in patients with Alzheimer's disease. J Neurol Sci 78:151-164.

Davies P, Maloney AJ (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 2:1403.

Dechant G, Barde YA (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. Nat Neurosci 5:1131-1136.

Deshpande A, Mina E, Glabe C, Busciglio J (2006) Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons. J Neurosci 26:6011-6018.

Dodge ME, Wang J, Guy C, Rankin S, Rahimtula M, Mearow KM (2006) Stress-induced heat shock protein 27 expression and its role in dorsal root ganglion neuronal survival. Brain Res 1068:34-48.

Dou F, Netzer WJ, Tanemura K, Li F, Hartl FU, Takashima A, Gouras GK, Greengard P, Xu H (2003) Chaperones increase association of tau protein with microtubules. Proc Natl Acad Sci U S A 100:721-726.

Evans CG, Wisen S, Gestwicki JE (2006) Heat shock proteins 70 and 90 inhibit early stages of amyloid beta-(1-42) aggregation in vitro. J Biol Chem 281:33182-33191.

Evgrafov OV, Mersiyanova I, Irobi J, Van Den Bosch L, Dierick I, Leung CL, Schagina O, Verpoorten N, Van Impe K, Fedotov V, Dadali E, Auer-Grumbach M, Windpassinger C, Wagner K, Mitrovic Z, Hilton-Jones D, Talbot K, Martin JJ, Vasserman N, Tverskaya S, Polyakov A, Liem RK, Gettemans J, Robberecht W, De Jonghe P, Timmerman V (2004) Mutant small heat-shock protein 27 causes

axonal Charcot-Marie-Tooth disease and distal hereditary motor neuropathy. Nat Genet 36:602-606.

Frolich L (2002) The cholinergic pathology in Alzheimer's disease--discrepancies between clinical experience and pathophysiological findings. J Neural Transm 109:1003-1013.

Gabuzda D, Busciglio J, Yankner BA (1993) Inhibition of beta-amyloid production by activation of protein kinase C. J Neurochem 61:2326-2329.

Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP, Solary E (1999) HSP27 inhibits cytochrome c-dependent activation of procaspase-9. Faseb J 13:2061-2070.

Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, Landry J (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci 110 (Pt 3):357-368.

Gusev NB, Bogatcheva NV, Marston SB (2002) Structure and properties of small heat shock proteins (sHsp) and their interaction with cytoskeleton proteins. Biochemistry (Mosc) 67:511-519.

Hamos JE, Oblas B, Pulaski-Salo D, Welch WJ, Bole DG, Drachman DA (1991) Expression of heat shock proteins in Alzheimer's disease. Neurology 41:345-350.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-356.

Hargis MT, Storck CW, Wickstrom E, Yakubov LA, Leeper DB, Coss RA (2004) Hsp27 anti-sense oligonucleotides sensitize the microtubular cytoskeleton of Chinese hamster ovary cells grown at low pH to 42 degrees C-induced reorganization. Int J Hyperthermia 20:491-502.

Harper JD, Wong SS, Lieber CM, Lansbury PT, Jr. (1999) Assembly of A beta amyloid protofibrils: an in vitro model for a possible early event in Alzheimer's disease. Biochemistry 38:8972-8980.

Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, Selkoe DJ (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. J Neurosci 19:8876-8884.

Havasi A, Li Z, Wang Z, Martin JL, Botla V, Ruchalski K, Schwartz JH, Borkan SC (2008) Hsp27 inhibits Bax activation and apoptosis via a phosphatidylinositol 3-kinase-dependent mechanism. J Biol Chem 283:12305-12313.

Heredia L, Lin R, Vigo FS, Kedikian G, Busciglio J, Lorenzo A (2004) Deposition of amyloid fibrils promotes cell-surface accumulation of amyloid beta precursor protein. Neurobiol Dis 16:617-629.

Hightower LE (1991) Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 66:191-197.

Hino M, Kurogi K, Okubo MA, Murata-Hori M, Hosoya H (2000) Small heat shock protein 27 (HSP27) associates with tubulin/microtubules in HeLa cells. Biochem Biophys Res Commun 271:164-169.

Huot J, Houle F, Spitz DR, Landry J (1996) HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. Cancer Res 56:273-279.

Ingolia TD, Slater MR, Craig EA (1982) Saccharomyces cerevisiae contains a complex multigene family related to the major heat shock-inducible gene of Drosophila. Mol Cell Biol 2:1388-1398.

Irobi J, Van Impe K, Seeman P, Jordanova A, Dierick I, Verpoorten N, Michalik A, De Vriendt E, Jacobs A, Van Gerwen V, Vennekens K, Mazanec R, Tournev I, Hilton-Jones D, Talbot K, Kremensky I, Van Den Bosch L, Robberecht W, Van Vandekerckhove J, Van Broeckhoven C, Gettemans J, De Jonghe P, Timmerman V (2004) Hot-spot residue in small heat-shock protein 22 causes distal motor neuropathy. Nat Genet 36:597-601.

Jellinger K, Danielczyk W, Fischer P, Gabriel E (1990) Clinicopathological analysis of dementia disorders in the elderly. J Neurol Sci 95:239-258.

Kamal A, Stokin GB, Yang Z, Xia CH, Goldstein LS (2000) Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron 28:449-459.

Kato K, Shinohara H, Kurobe N, Inaguma Y, Shimizu K, Ohshima K (1991) Tissue distribution and developmental profiles of immunoreactive alpha B crystallin in the rat determined with a sensitive immunoassay system. Biochim Biophys Acta 1074:201-208.

Kawahara M, Kuroda Y (2000) Molecular mechanism of neurodegeneration induced by Alzheimer's beta-amyloid protein: channel formation and disruption of calcium homeostasis. Brain Res Bull 53:389-397.

Kim HJ, Chae SC, Lee DK, Chromy B, Lee SC, Park YC, Klein WL, Krafft GA, Hong ST (2003) Selective neuronal degeneration induced by soluble oligomeric amyloid beta protein. Faseb J 17:118-120.

Kim KK, Kim R, Kim SH (1998) Crystal structure of a small heat-shock protein. Nature 394:595-599.

Kinoshita A, Whelan CM, Berezovska O, Hyman BT (2002) The gamma secretase-generated carboxyl-terminal domain of the amyloid precursor protein induces apoptosis via Tip60 in H4 cells. J Biol Chem 277:28530-28536.

Klein WL (2002) Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. Neurochem Int 41:345-352.

Konishi H, Matsuzaki H, Tanaka M, Takemura Y, Kuroda S, Ono Y, Kikkawa U (1997) Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27. FEBS Lett 410:493-498.

Krueger AM, Armstrong JN, Plumier J, Robertson HA, Currie RW (1999) Cell specific expression of Hsp70 in neurons and glia of the rat hippocampus after hyperthermia and kainic acid-induced seizure activity. Brain Res Mol Brain Res 71:265-278.

Kuner P, Schubenel R, Hertel C (1998) Beta-amyloid binds to p57NTR and activates NFkappaB in human neuroblastoma cells. J Neurosci Res 54:798-804.

Lambert H, Charette SJ, Bernier AF, Guimond A, Landry J (1999) HSP27 multimerization mediated by phosphorylation-sensitive intermolecular interactions at the amino terminus. J Biol Chem 274:9378-9385.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A 95:6448-6453.

Lavoie JN, Hickey E, Weber LA, Landry J (1993) Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J Biol Chem 268:24210-24214.

Lee JS, Zhang MH, Yun EK, Geum D, Kim K, Kim TH, Lim YS, Seo JS (2005) Heat shock protein 27 interacts with vimentin and prevents insolubilization of vimentin subunits induced by cadmium. Exp Mol Med 37:427-435.

Lelj-Garolla B, Mauk AG (2005) Self-association of a small heat shock protein. J Mol Biol 345:631-642.

Lorenzo A, Yankner BA (1994) Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. Proc Natl Acad Sci U S A 91:12243-12247.

Maccioni RB, Munoz JP, Barbeito L (2001) The molecular bases of Alzheimer's disease and other neurodegenerative disorders. Arch Med Res 32:367-381.

Magrane J, Smith RC, Walsh K, Querfurth HW (2004) Heat shock protein 70 participates in the neuroprotective response to intracellularly expressed beta-amyloid in neurons. J Neurosci 24:1700-1706.

Magrane J, Rosen KM, Smith RC, Walsh K, Gouras GK, Querfurth HW (2005) Intraneuronal beta-amyloid expression downregulates the Akt survival pathway and blunts the stress response. J Neurosci 25:10960-10969.

Mailhos C, Howard MK, Latchman DS (1994) Heat shock proteins hsp90 and hsp70 protect neuronal cells from thermal stress but not from programmed cell death. J Neurochem 63:1787-1795.

Manzerra P, Rush SJ, Brown IR (1997) Tissue-specific differences in heat shock protein hsc70 and hsp70 in the control and hyperthermic rabbit. J Cell Physiol 170:130-137.

Masliah E, Sisk A, Mallory M, Mucke L, Schenk D, Games D (1996) Comparison of neurodegenerative pathology in transgenic mice overexpressing V717F beta-amyloid precursor protein and Alzheimer's disease. J Neurosci 16:5795-5811.

Mattson MP (2004) Pathways towards and away from Alzheimer's disease. Nature 430:631-639.

Mattson MP, Magnus T (2006) Ageing and neuronal vulnerability. Nat Rev Neurosci 7:278-294.

Mearow KM, Dodge ME, Rahimtula M, Yegappan C (2002) Stress-mediated signaling in PC12 cells - the role of the small heat shock protein, Hsp27, and Akt in protecting cells from heat stress and nerve growth factor withdrawal. J Neurochem 83:452-462.

Meberg PJ, Bamburg JR (2000) Increase in neurite outgrowth mediated by overexpression of actin depolymerizing factor. J Neurosci 20:2459-2469.

Meyer MR, Tschanz JT, Norton MC, Welsh-Bohmer KA, Steffens DC, Wyse BW, Breitner JC (1998) APOE genotype predicts when--not whether--one is predisposed to develop Alzheimer disease. Nat Genet 19:321-322.

Michaelis ML, Seyb KI, Ansar S (2005) Cytoskeletal integrity as a drug target. Curr Alzheimer Res 2:227-229.

Miron T, Vancompernolle K, Vandekerckhove J, Wilchek M, Geiger B (1991) A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J Cell Biol 114:255-261.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55-63.

Mounier N, Arrigo AP (2002) Actin cytoskeleton and small heat shock proteins: how do they interact? Cell Stress Chaperones 7:167-176.

Muchowski PJ, Wacker JL (2005) Modulation of neurodegeneration by molecular chaperones. Nat Rev Neurosci 6:11-22.

Muth K, Schonmeyer R, Matura S, Haenschel C, Schroder J, Pantel J (2009) Mild Cognitive Impairment in the Elderly is Associated with Volume Loss of the Cholinergic Basal Forebrain Region. Biol Psychiatry.

Oddo S, Billings L, Kesslak JP, Cribbs DH, LaFerla FM (2004) Abeta immunotherapy leads to clearance of early, but not late, hyperphosphorylated tau aggregates via the proteasome. Neuron 43:321-332.

Ohshima Y, Kubo T, Koyama R, Ueno M, Nakagawa M, Yamashita T (2008) Regulation of axonal elongation and pathfinding from the entorhinal cortex to the dentate gyrus in the hippocampus by the chemokine stromal cell-derived factor 1 alpha. J Neurosci 28:8344-8353.

Pappas BA, Bayley PJ, Bui BK, Hansen LA, Thal LJ (2000) Choline acetyltransferase activity and cognitive domain scores of Alzheimer's patients. Neurobiol Aging 21:11-17.

Parsell DA, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet 27:437-496.

Patel YJ, Payne Smith MD, de Belleroche J, Latchman DS (2005) Hsp27 and Hsp70 administered in combination have a potent protective effect against FALS-associated SOD1-mutant-induced cell death in mammalian neuronal cells. Brain Res Mol Brain Res 134:256-274.

Perez-Olle R, Jones ST, Liem RK (2004) Phenotypic analysis of neurofilament light gene mutations linked to Charcot-Marie-Tooth disease in cell culture models. Hum Mol Genet 13:2207-2220.

Perng MD, Cairns L, van den IP, Prescott A, Hutcheson AM, Quinlan RA (1999) Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin. J Cell Sci 112 (Pt 13):2099-2112.

Pike CJ, Overman MJ, Cotman CW (1995) Amino-terminal deletions enhance aggregation of beta-amyloid peptides in vitro. J Biol Chem 270:23895-23898.

Pike CJ, Burdick D, Walencewicz AJ, Glabe CG, Cotman CW (1993) Neurodegeneration induced by beta-amyloid peptides in vitro: the role of peptide assembly state. J Neurosci 13:1676-1687.

Poorkaj P, Sharma V, Anderson L, Nemens E, Alonso ME, Orr H, White J, Heston L, Bird TD, Schellenberg GD (1998) Missense mutations in the chromosome 14 familial Alzheimer's disease presenilin 1 gene. Hum Mutat 11:216-221.

Price DL, Sisodia SS, Borchelt DR (1998) Alzheimer disease--when and why? Nat Genet 19:314-316.

Renkawek K, Bosman GJ, Gaestel M (1993) Increased expression of heat-shock protein 27 kDa in Alzheimer disease: a preliminary study. Neuroreport 5:14-16.

Renkawek K, Bosman GJ, de Jong WW (1994) Expression of small heat-shock protein hsp 27 in reactive gliosis in Alzheimer disease and other types of dementia. Acta Neuropathol 87:511-519.

Resende R, Pereira C, Agostinho P, Vieira AP, Malva JO, Oliveira CR (2007) Susceptibility of hippocampal neurons to Abeta peptide toxicity is associated with perturbation of Ca2+ homeostasis. Brain Res 1143:11-21.

Ritossa F (1962) A new puffing pattern induced by a temperature shock and DNP in Drosophilia. Experientia 18: 571-573.

Rogalla T, Ehrnsperger M, Preville X, Kotlyarov A, Lutsch G, Ducasse C, Paul C, Wieske M, Arrigo AP, Buchner J, Gaestel M (1999) Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative

stress/tumor necrosis factor alpha by phosphorylation. J Biol Chem 274:18947-18956.

Scheff SW, DeKosky ST, Price DA (1990) Quantitative assessment of cortical synaptic density in Alzheimer's disease. Neurobiol Aging 11:29-37.

Sharp P, Krishnan M, Pullar O, Navarrete R, Wells D, de Belleroche J (2006) Heat shock protein 27 rescues motor neurons following nerve injury and preserves muscle function. Exp Neurol 198:511-518.

Shea TB, Dahl DC, Nixon RA, Fischer I (1997) Triton-soluble phosphovariants of the heavy neurofilament subunit in developing and mature mouse central nervous system. J Neurosci Res 48:515-523.

Shimura H, Miura-Shimura Y, Kosik KS (2004) Binding of tau to heat shock protein 27 leads to decreased concentration of hyperphosphorylated tau and enhanced cell survival. J Biol Chem 279:17957-17962.

Song C, Perides G, Wang D, Liu YF (2002) beta-Amyloid peptide induces formation of actin stress fibers through p38 mitogen-activated protein kinase. J Neurochem 83:828-836.

Sotthibundhu A, Sykes AM, Fox B, Underwood CK, Thangnipon W, Coulson EJ (2008) Beta-amyloid(1-42) induces neuronal death through the p75 neurotrophin receptor. J Neurosci 28:3941-3946.

Stege GJ, Renkawek K, Overkamp PS, Verschuure P, van Rijk AF, Reijnen-Aalbers A, Boelens WC, Bosman GJ, de Jong WW (1999) The molecular chaperone alphaB-crystallin enhances amyloid beta neurotoxicity. Biochem Biophys Res Commun 262:152-156.

Sugiyama Y, Suzuki A, Kishikawa M, Akutsu R, Hirose T, Waye MM, Tsui SK, Yoshida S, Ohno S (2000) Muscle develops a specific form of small heat shock protein complex composed of MKBP/HSPB2 and HSPB3 during myogenic differentiation. J Biol Chem 275:1095-1104.

Takahashi RH, Capetillo-Zarate E, Lin MT, Milner TA, Gouras GK (2008) Co-occurrence of Alzheimer's disease beta-amyloid and tau pathologies at synapses. Neurobiol Aging.

Takai S, Matsushima-Nishiwaki R, Tokuda H, Yasuda E, Toyoda H, Kaneoka Y, Yamaguchi A, Kumada T, Kozawa O (2007) Protein kinase C delta regulates the phosphorylation of heat shock protein 27 in human hepatocellular carcinoma. Life Sci 81:585-591.

Tanzi RE, Bertram L (2001) New frontiers in Alzheimer's disease genetics. Neuron 32:181-184.

Taylor RP, Benjamin IJ (2005) Small heat shock proteins: a new classification scheme in mammals. J Mol Cell Cardiol 38:433-444.

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 30:572-580.

Tomozawa Y, Appel SH (1986) Soluble striatal extracts enhance development of mesencephalic dopaminergic neurons in vitro. Brain Res 399:111-124.

Toni N, Buchs PA, Nikonenko I, Bron CR, Muller D (1999) LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. Nature 402:421-425.

Townsend M, Shankar GM, Mehta T, Walsh DM, Selkoe DJ (2006) Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers. J Physiol 572:477-492.

Troy CM, Friedman JE, Friedman WJ (2002) Mechanisms of p75-mediated death of hippocampal neurons. Role of caspases. J Biol Chem 277:34295-34302.

Venkitaramani DV, Chin J, Netzer WJ, Gouras GK, Lesne S, Malinow R, Lombroso PJ (2007) Beta-amyloid modulation of synaptic transmission and plasticity. J Neurosci 27:11832-11837.

Vicart P, Caron A, Guicheney P, Li Z, Prevost MC, Faure A, Chateau D, Chapon F, Tome F, Dupret JM, Paulin D, Fardeau M (1998) A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. Nat Genet 20:92-95.

Walsh DM, Selkoe DJ (2004) Deciphering the molecular basis of memory failure in Alzheimer's disease. Neuron 44:181-193.

Whalen BM, Selkoe DJ, Hartley DM (2005) Small non-fibrillar assemblies of amyloid beta-protein bearing the Arctic mutation induce rapid neuritic degeneration. Neurobiol Dis 20:254-266.

Williams KL, Rahimtula M, Mearow KM (2005) Hsp27 and axonal growth in adult sensory neurons in vitro. BMC Neurosci 6:24.

Williams KL, Rahimtula M, Mearow KM (2006) Heat shock protein 27 is involved in neurite extension and branching of dorsal root ganglion neurons in vitro. J Neurosci Res 84:716-723.

Wirths O, Breyhan H, Schafer S, Roth C, Bayer TA (2008) Deficits in working memory and motor performance in the APP/PS1ki mouse model for Alzheimer's disease. Neurobiol Aging 29:891-901.

Yaar M, Zhai S, Pilch PF, Doyle SM, Eisenhauer PB, Fine RE, Gilchrest BA (1997) Binding of beta-amyloid to the p75 neurotrophin receptor induces apoptosis. A possible mechanism for Alzheimer's disease. J Clin Invest 100:2333-2340.

Yankner BA, Caceres A, Duffy LK (1990) Nerve growth factor potentiates the neurotoxicity of beta amyloid. Proc Natl Acad Sci U S A 87:9020-9023.

Zheng P, Eastman J, Vande Pol S, Pimplikar SW (1998) PAT1, a microtubule-interacting protein, recognizes the basolateral sorting signal of amyloid precursor protein. Proc Natl Acad Sci U S A 95:14745-14750.

Appendix 1

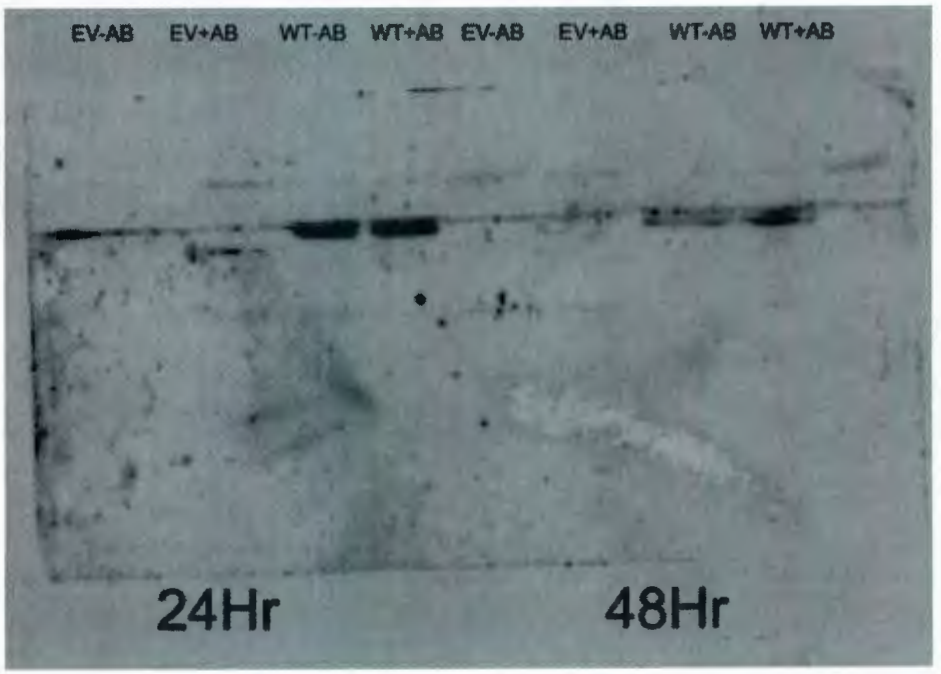


Figure 5.1. Western Blot shows undetectable levels of endogenous Hsp27 in untreated and $A\beta_{1-42}$ treated conditions. Cortical neurons were plated on poly-D-lysine coated dishes, allowed to mature for two days in culture and then treated with $A\beta_{1-42}$ for 24 and 48 hr. Although Hsp27WT fusion protein is present, it can be seen that endogenous Hsp27 is not detectable in any experimental condition.