Mitochondrial Heat Shock Protein 60: Evaluation of its role as a neuroprotectant in familial ALS and its mutation as a cause of hereditary spastic paraplegia

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ABSTRACT

In the fatal neurodegenerative disease, amyotrophic lateral sclerosis (ALS), protein misfolding and aggregation are involved in motor neuron death. Heat shock proteins (Hsp), which help refold misfolded proteins or target them to the proteasome for degradation, have been studied as a therapeutic target in ALS, with some success. At the same time, it has been shown that mitochondrial dysfunction is one of the earliest pathogenic events leading to motor neuron death in ALS. The mitochondria have their own Hsps, for protein folding in the matrix, an important example of which is Hsp60. Given the finding that increased expression of another mitochondrial Hsp, Hsp22, preserved motor function, reduced motor neuron death and increased resistance to oxidative stress in a *Drosophila* model of aging, it is possible that the upregulation of mitochondrial Hsp60 might have similar neuroprotective effects in ALS. The current study examined Hsp60 upregulation in a primary dissociated spinal cord culture model of familial ALS (fALS) due to mutation in the *SOD1* gene. Increasing Hsp60 expression failed to improve motor neuron viability, to ameliorate SOD1 protein misfolding/aggregation, and to prevent mitochondrial rounding in motor neurons. Therefore, the upregulation of mitochondrial Hsp60 would not be an effective therapy for ALS.

Meanwhile, dominantly-inherited mutations in the gene encoding for Hsp60 have been linked to hereditary spastic paraplegia SPG13 (HSPG13), a disease in which the axons of motor neurons degenerate. Disease mechanisms are not known; however, given

the importance of mitochondria to axonal integrity, and the mitochondrial localization of Hsp60, mitochondrial dysfunction seems a likely candidate. $Hsp60^{V72I}$, a mutation found in HSPG13 patients, was expressed in motor neurons in primary dissociated spinal cord culture to model potential mitochondrial abnormalities. $Hsp60^{V72I}$ expression did not reduce motor neuron viability, alter mitochondrial morphology, or impair mitochondria on functional measures, including membrane potential ($\Delta\Psi$), resistance to oxidative stress, or axonal transport. It is possible that $Hsp60^{V72I}$ causes no, or a weak, mitochondrial phenotype. However, the expression of a severe, ATPase-deficient mutant, $Hsp60^{D423A}$, also failed to induce mitochondrial dysfunction in this culture model. Thus, these phenotypes might not manifest because of culture conditions or because they are masked by the high level of endogenous Hsp60.

RÉSUMÉ

Lors de la maladie neurodégénérative de la sclérose latérale amyotrophique (SLA), le mauvais repliement et l'agrégation des protéines ont une incidence sur la mort des motoneurones. Les protéines du choc thermique (Hsp), lesquelles aident les protéines au mauvais repliement à se replier de nouveau ou encore ciblent ces dernières au niveau du protéasome pour leur dégradation, ont été étudiées comme source de traitement possible pour la SLA, non sans réussite. Parallèlement, il a été démontré que le dysfonctionnement mitochondrial est un des événements pathogènes menant à la mort des motoneurones, les anomalies mitochondriales apparaissant avant qu'une agrégation ou un mauvais repliement des protéines significatif ne soit observé. Les mitochondries ont leurs propres Hsp pour les protéines se repliant dans la matrice, les Hsp60 étant l'exemple par excellence. Si on considère que l'expression intensifiée d'autres mitochondries comme les Hsp22 peut préserver la fonction motrice, empêcher la mort de motoneurones et augmenter la résistance au stress oxydatif dans un modèle vieillissant de *Drosophila,* il semble que la régulation positive des Hsp60 mitochondriales pourrait possiblement protéger les neurones contre la SLA. La présente étude avait pour but d'examiner la régulation positive des Hsp60 dans un modèle de moelle épinière dissociée atteinte de SLA familiale causée par une mutation du gène *SOD1*. L'intensification de l'expression des Hsp60 n'a pas contribué à préserver la viabilité des motoneurones, ni à rectifier le mauvais repliement ou l'agrégation des protéines SOD1, ni à prévenir

l'arrondissement mitochondrial dans les motoneurones. Par conséquent, la régulation positive des Hsp60 mitochondriales n'est pas recommandée pour le traitement de la SLA.

Entretemps, les mutations de l'encodage du gène pour les Hsp60 transmises selon le mode dominant ont été associées à la paraplégie spasmodique héréditaire SPG13 (HSPG13), une maladie au cours de laquelle la dégénérescence axonique cause la mort des motoneurones. On ne connait aucun autre mécanisme de mort cellulaire plus précis. Toutefois, étant donné la présence des anomalies mitochondriales dans grand nombre de maladies neurodégénératives, l'importance des mitochondries pour l'intégrité des axones et la localisation mitochondriale des Hsp60, le dysfonctionnement mitochondrial pourrait très bien constituer un tel mécanisme. Les $Hsp60^{V72I}$, une mutation observée chez les patients atteints de HSPG13, ont pu être exprimées dans les motoneurones provenant de cultures de moelle épinière dissociée afin de servir de modèle pour de potentielles anomalies mitochondriales. L'expression des $Hsp60^{V72I}$ n'a pas mené à une baisse de viabilité chez les motoneurones, ni à une modification de la morphologie mitochondriale, ni à une détérioration des mesures fonctionnelles des mitochondries, incluant le potentiel de membrane ($\Delta\Psi$), la résistance au stress oxydatif et le transport axonal. Il se peut que les $Hsp60^{V72I}$ ne causent qu'un faible phénotype mitochondrial, ou encore qu'elles n'en causent aucun du tout. Cependant, l'expression d'un mutant à grave déficience en ATP-ase, soit les $Hsp60^{D423A}$, n'est pas non plus parvenue à causer un dysfonctionnement mitochondrial dans ce modèle de culture. On ne sait toujours pas si l'absence de phénotype est révélatrice du rôle des Hsp60 dans les maladies touchant les motoneurones, ou si les résultats négatifs sont un artefact du modèle de culture, lequel était caractérisé par un niveau élevé d'expression endogène des Hsp60 de phénotype sauvage qui pourrait

potentiellement masquer les effets de l'expression du mutant exogène Hsp60. Les études à venir devraient se pencher sur les effets mitochondriaux de l'expression des Hsp60^{V72I} dans un contexte où le phénotype sauvage Hsp60 endogène est réduit, en utilisant par exemple les Hsp60 shRNA particulières aux souris.

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LIST OF FIGURES

ABBREVIATIONS

°C	degrees Celsius
Δψ	mitochondrial membrane potential
μg	microgram
μL	microliter
μm	micrometer
μM	micromolar
ηg	nanogram
ηM	nanomolar
ADP	adenosine diphosphate
ALS	amyotrophic lateral sclerosis
ANG	angiogenin
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	enzyme that catalyzes ATP hydrolysis
Bcl-2	B-cell leukemia/lymphoma 2 anti-apoptotic protein
BSSG	β-sitosterol β-D-glucoside
cDNA	complimentary DNA
CNS	central nervous system
CO_2	carbon dioxide
Cy2	cyanine 2

Cy3	cyanine 3
Cy5	cyanine 5
Cyt-c	cytochrome-c
D423A	aspartic acid to alanine substitution at position 423 of human Hsp60
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
dsRED	Discosoma red fluorescent protein
E13	embryonic day 13
eGFP	enhanced green fluorescent protein
EMEM	Eagle's minimum essential medium
ER	endoplasmic reticulum
fALS	familial amyotrophic lateral sclerosis
FITC	fluorescent isothiocyanate
FUS/TLS	fused in sarcoma/translated in liposarcoma
G37R	glycine to arginine substitution at position 37 of mouse SOD1
G85R	glycine to arginine substitution at position 85 of mouse SOD1
G93A	glycine to alanine substitution at position 93 of mouse or human SOD1
g	grams
GroEL	60 kDa heat shock protein of *E. coli*
GroES	10 kDa heat shock co-chaperone of *E. coli*
GSH	reduced glutathione

H_2O_2	hydrogen peroxide
H46R/H48Q	histidine to arginine/glutamine substitution at position 46/48 of mouse SOD1
Hb9	motor neuron specific transcription factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS	horse serum
Hsp	heat shock protein
Hsp60	60 kDa heat shock protein
HSPD1	gene encoding 60 kDa heat shock protein
HSPG13	hereditary spastic paraplegia type SPG13
IMS	intermembrane space of the mitochondria
iNOS	inducible nitric oxide synthase
KCl	potassium chloride
kDa	kilodalton
KH_2PO_4	potassium dihydrogen phosphate
L	litres
LSM	laser scanning microscopy
MEM	minimum essential medium
mg	milligrams
min	minutes
mL	milliliter
mm	millimeter
mM	millimolar

NA	numerical aperture
Na_2HPO_4	sodium hydrogen phosphate
NaCl	sodium chloride
$NaHCO_3$	bicarbonate
NFH	neurofilament heavy subunit
NIH	National Institutes of Health
NP-49	nonidet phenoxypolyethoxyethanol-40
O_2	oxygen
PF	3% paraformaldehyde
PBS	phosphate buffered saline
pOCTeGFP	eGFP with mitochondrial localization sequence
ROS	reactive oxygen species
sALS	sporadic ALS
SMI32	antibody against non-phosphorylated neurofilaments
SMN	survival motor neuron protein
SNP	single nucleotide polymorphism
SOD1	copper/zinc superoxide dismutase-1
SPG7	hereditary spastic paraplegia type 7
SPG13	hereditary spastic paraplegia type 13
SPSS	Statistical Package for Social Sciences
TDP-43	TAR-DNA binding protein-43
TLR4	toll-like receptor 4
TMRM	tetramethyl rhodamine methyl ester

UPR	unfolded protein response
V72I	valine to isoleucine substitution at position 72 of human Hsp60
V98I	valine to isoleucine substitution at position 98 of human Hsp60
VAPB	vesicle-associated membrane protein/synaptobrevin-associated membrane protein B
VDAC1	voltage-dependent anion channel 1
VEGF	vascular endothelial growth factor
WT	wild-type

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) and hereditary spastic paraplegia are diseases that affect the function and integrity of neurons in the central nervous system (CNS) (de Carvalho et al., 2008, Donaghy, 1999). ALS affects neurons of the cortex, brainstem and spinal cord, and the death of upper and lower motor neurons causes progressive muscle atrophy and weakness of upper and lower limbs, or with the more pernicious bulbar onset, the muscles that control speech and swallowing (Mitchell and Borasio, 2007). 90% of ALS cases are sporadic (sALS), meaning that they have no clear genetic cause. The remaining 10% of cases are hereditary or familial (fALS) (Cozzolino et al., 2008). Of the genes known to be mutated to cause ALS, the gene encoding Cu/Zn superoxide dismutase (SOD1) (Rosen, 1993), is the best studied, and is the focus of the first hypothesis for this work. ALS is invariably fatal, with most patients dying of respiratory failure. Hereditary spastic paraplegia, while not fatal, features progressive lower limb spasticity and weakness caused by the loss of motor neuron axons in the spinal cord. The inheritance of the disease has been linked to at least 17 genes, and diagnosis is specified by subtype, based on the particular gene mutation (Salinas et al., 2008). For the second hypothesis for this work, a subtype of hereditary spastic paraplegia, HSPG13, caused by mutation in the gene encoding the mitochondrial heat shock protein Hsp60 (Hansen et al., 2002, Hansen et al., 2007, Bross et al., 2008), is being studied.

A prominent feature of ALS, including fALS due to mutation in SOD1, is protein misfolding and aggregation, which is associated with motor neuron death (Goldberg, 2003). The primary dissociated spinal cord culture model of fALS due to the $SOD1^{G93A}$

mutation employed for this work mirrors this feature of protein (mutant SOD1) folding pathology and neuronal death (Durham et al., 1997). An attractive target for the amelioration of protein misfolding is the heat shock proteins (Hsp), which act as protein chaperones, binding and stabilizing misfolded proteins to assist with refolding, or targeting aberrant proteins for degradation by the proteasome (Nollen and Morimoto, 2002).

Another feature of ALS pathogenesis is mitochondrial abnormalities, which are observed at early time points, even before protein misfolding and aggregation are observed (Tradewell et al., 2009). However, mitochondrial pathology is not limited to ALS, but is a common link between many neurodegenerative diseases, for example Alzheimer's disease, Parkinson's disease, and Charcot-Marie-Tooth disease (Lin and Beal, 2006). In animal and culture models of fALS due to the $SOD1^{G93A}$ mutation, mutant, misfolded SOD1 is seen to associate inappropriately with mitochondria, demonstrating a link between protein misfolding and mitochondrial dysfunction (Liu et al., 2004, Pasinelli et al., 2004, Vijayvergiya, 2005, Vande Velde, 2008, Israelson et al., 2010, Pedrini et al., 2010). Furthermore, the mitochondria have their own set of Hsps, which could potentially be up-regulated to help protect the mitochondria from damage early in the course of disease. Hypothesis 1 of the current work specifically examines the up-regulation of the mitochondrial heat shock protein, Hsp60, as a means of protecting motor neurons and their mitochondria in a primary dissociated spinal cord culture model of fALS due to $SOD1^{G93A}$ expression.

Tying together the concepts of mitochondrial dysfunction in neurodegenerative disease and the function of mitochondrial heat shock proteins in neuronal protection is

the finding that mutations in the gene encoding mitochondrial Hsp60 are associated with HSPG13 (Hansen et al., 2002, Hansen et al., 2007, Bross et al., 2008). Hypothesis 2 of the current work examines the expression of the disease-associated mutant protein, $Hsp60^{V72I}$, to create a primary dissociated spinal cord culture model of HSPG13, potentially elucidating specific mitochondrial abnormalities that may be present in HSPG13.

By examining Hsp60 as a potential protector of motor neurons via a mitochondrial pathway in a culture model of SOD1-associated fALS (hypothesis 1), and, on the other hand, examining how mutation in Hsp60 might lead to mitochondrial dysfunction in a culture model of HSPG13 (hypothesis 2), it was hoped to unearth key information about the function of Hsp60 in motor neurons more generally, and to elucidate potential common pathways of mitochondrial dysfunction that contribute to multiple neurodegenerative diseases.

CHAPTER 1 –Literature Review

1.1 Amyotrophic Lateral Sclerosis

1.1.1 Clinical Overview

Amyotrophic Lateral Sclerosis, a.k.a. Lou Gehrig's disease, is a late-onset degenerative disease affecting motor neurons of the cortex, brainstem and spinal cord. Loss of upper and lower motor neurons causes muscle weakness and atrophy. Onset may be bulbar, affecting the muscles involved in speech and swallowing; cervical, impairing movement of arms and shoulders; or lumbar, making walking difficult. These symptoms reflect the location of the motor neurons most affected in each patient (Mitchell and Borasio, 2007). However, though motor neurons are selectively vulnerable in ALS, the disease is considered to be non-cell-autonomous, meaning that involvement of cell types other than motor neurons, especially glial cells, is necessary in causing the death of motor neurons (Gong et al., 2000, Pramatarova et al., 2001, Clement et al., 2003, Boillee et al., 2006b). Disease prevalence is 4-6 per 100,000 people and incidence is 1-2 per 100,000. Diagnosis is based on El Escorial World Federation of Neurology criteria (Brooks, 1994), which identify the progressive, specific loss of upper and lower motor neurons, while ruling out clinical symptoms of other diseases (de Carvalho et al., 2008). Though clinically indistinguishable, ALS cases are classified as familial (fALS) or sporadic (sALS), depending on whether or not the disease is inherited and linked to one of the known genetic mutations causing the disease (Boillee et al., 2006b). Patients are usually diagnosed in the fifth or sixth decade of life and on average die of respiratory failure 1-5 years after initial diagnosis. At present, there is no cure for ALS, and the few treatments

available, for example, riluzole, are relatively ineffective (Cozzolino et al., 2008, Costa et al., 2010).

1.1.2 Sporadic ALS

The vast majority (about 90%) of ALS cases are classified as sporadic. A number of environmental and genetic components have been implicated in sALS.

One environmental factor with a strong link to a particular variety of ALS on Guam is the consumption of flour made from cycad seeds, first associated with a sALS endemic in Guam, typically with additional features of Parkinson's and dementia (Kisby et al., 1992). A likely candidate for the toxic component of cycad flour is non-water-soluble phytosterol β-D-glucosides, especially β-sitosterol β-D-glucoside (BSSG), which causes neuronal death by glutamate excitotoxicity in cortical slices and primary cortical cultures (Khabazian et al., 2002).

Exposure to heavy metals may pose a heightened risk for ALS, but the literature on this remains controversial. A low calcium/magnesium, high aluminum diet caused loss of neurons in the frontal and parietal cortices, as well as the spinal cord in mice (Kihira et al., 2002). Occupational lead exposure may also slightly increase the risk for ALS (Kamel et al., 2005, Qureshi et al., 2006).

Chemical agents used in warfare, especially the Gulf War (Rose and Brix, 2006), and pesticides (Qureshi et al., 2006) and insecticides used in agriculture are putative environmental risks for sALS, but firm conclusions are difficult to draw, as high-quality studies are lacking (Sutedja et al., 2009).

A meta-analysis of over a decade of environmental risk factors identified smoking as a "probable risk factor", while identifying trauma, physical activity, rural residency, and alcohol consumption as improbable risk factors (Armon, 2003).

It is likely that environmental risk factors interact with genetic risk factors to increase predisposition to disease. A number of genetic factors are associated with sALS (reviewed in Schymick et al., 2007). The list of possible genetic contributors to sALS includes, but is not limited to: single nucleotide polymorphism (SNP) in the vascular endothelial growth factor (VEGF) gene in male carriers (Lambrechts et al., 2009), loss of function mutations in the gene encoding angiogenin (ANG) (Kishikawa et al., 2008), SNPs in the paraoxonase genes (Ticozzi et al., 2010) (though this association has been disputed; see Wills et al., 2009), mutations in the neurofilament heavy subunit (NFH) (Al-Chalabi et al., 1999), reduced survival motor neuron proteins (SMN1 and possibly 2) (Veldink et al., 2005, Corcia et al., 2006), mutations in HSPB1 leading to impaired Hsp27 stress response (Dierick et al., 2007), and mutations in the vesicle-associated membrane protein/synaptobrevin-associated membrane protein B (VAPB) (Nishimura et al., 2004).

Clearly, better-validated, controlled studies of environmental and genetic risk factors, and their possible interactions, are needed to better understand their contribution to sALS etiology.

1.1.3 Familial ALS

10-20% of ALS cases are inherited (familial ALS; fALS) (Cozzolino et al., 2008). The strongest links to classical, inherited, adult-onset ALS are mutations in the genes encoding Cu/Zn superoxide dismutase (SOD1) (Rosen, 1993), TAR-DNA binding protein-43 (TARDBP; TDP-43) (Kabashi et al., 2008b), and fused in sarcoma/translated in liposarcoma (FUS/TLS) (Kwiatkowski et al., 2009). Of these, SOD1 is the best studied, and models of fALS due to SOD1 mutation have been used to unearth potential mechanisms of neuronal death. The current study models fALS by expressing a plasmid encoding the disease-causing $SOD1^{G93A}$ mutant in primary dissociated spinal cord culture prepared from embryonic mice; therefore, knowledge of the pathological manifestations of SOD1 mutation will be particularly relevant to understanding the experiments described in this thesis.

1.1.4 Cu/Zn Superoxide Dismutase (SOD1) Mutation and fALS

10-20% of fALS is due to mutations in SOD1 (Rosen, 1993), a homodimeric protein important in combatting oxidative stress by converting toxic superoxide, a byproduct of oxidative phosphorylation (Raha and Robinson, 2000), into O_2 and H_2O_2. Mutations occur in various regions of the 154-amino acid protein, with over 110 mutations to date having been linked to ALS (Valdmanis and Rouleau, 2008). A summary of the information available for all known ALS genes can be found at http://alsod.iop.kcl.ac.uk/Index.aspx.

fALS-causing mutations in SOD1 are said to confer a toxic gain-of-function to the enzyme (reviewed in Rothstein, 2009). Evidence for the gain-of-function hypothesis

includes the observation that knocking out SOD1 in mice failed to cause an ALS phenotype (Reaume et al., 1996), and the fact that many SOD1 mutations that cause ALS retain significant enzymatic activity (Borchelt et al., 1994). In mouse models of ALS expressing the $SOD1^{G93A}$ and $SOD1^{G37R}$ mutants, motor neurons degenerate despite a 4- to 14-fold increase in dismutase activity compared to non-transgenic littermates (Wong et al., 1995, Bruijn et al., 1997). On the other hand, the $SOD1^{G85R}$ mutation also causes ALS, but is enzymatically inactive (Bruijn et al., 1997). Therefore, it may be more accurate to posit that ALS pathogenesis results from a variety of mechanisms independent of changes in SOD1 dismutase activity. Indeed, ALS phenotype has been linked to mechanisms as diverse as oxidative stress, glutamate excitotoxicity, deficits in calcium handling, growth factor insufficiency, proteasome dysfunction, mitochondrial abnormalities, and protein misfolding and aggregation (reviewed in Boillee et al., 2006a, Rothstein, 2009). The latter two mechanisms are of particular interest to the present work, and will therefore be discussed in more detail.

1.1.5 Protein Aggregation in SOD1-Related fALS

An important pathogenic marker in many neurodegenerative diseases, including fALS due to SOD1 mutation, is the aggregation and accumulation of insoluble proteins into inclusions (Goldberg, 2003). SOD1-immunoreactive inclusions have been identified in the spinal cord tissue of fALS and sALS patients (Shibata et al., 1994, Shibata et al., 1996), in mouse and rat models of SOD1-related fALS (Bruijn et al., 1997, Aoki et al., 2005), and in cultures modeling SOD1 fALS using primary dissociated spinal cord from embryonic mice (Durham et al., 1997).

Whether SOD1 aggregation is causal, or just symptomatic, of toxicity to motor neurons remains a contentious issue; it is possible that SOD1 aggregates represent an end-point of failed protein quality control, rather than being the source of toxicity themselves. However, considerable evidence exists correlating misfolded, aggregated SOD1 with neurotoxicity. Matsumoto et al. (2005) found that SOD1 aggregate formation, due to expression of $SOD1^{G85R}$ or $SOD1^{G93A}$ in a neuronal cell line, was associated with a high rate of cell death (90% death after 48 hrs), whereas cells in the same cultures expressing diffuse mutant SOD1 had a 70% survival rate. Furthermore, mutant SOD1 aggregates sequestered proteasome, a potential mechanism by which protein aggregation could be neurotoxic. It is unlikely, however, that the entire proteasome is affected by mutant SOD1; indeed, other studies have detected proteasome inhibition and altered proteasome subunit composition, such as reduced beta subunits of the 20S proteasome core, without a reduction in overall proteasome, in mice expressing mutant SOD1(Kabashi et al., 2004, Cheroni et al., 2005, Kabashi et al., 2008a). In another study, misfolded, aggregated SOD1, either truncated $SOD1^{WT}$ or $SOD1^{A4V}$, was associated with apoptotic neuronal death in the spinal cords of embryonic chicks (Ghadge et al., 2006).

On the other hand, remedies that reduce SOD1 protein aggregation, for example by upregulating heat shock proteins to assist with protein folding, have been shown to increase the viability of cultured motor neurons (Bruening et al., 1999, Batulan et al., 2006) and transgenic mice (Kieran et al., 2004, Kiaei et al., 2005) expressing mutant SOD1.

In addition to the aforementioned proteasome subunit sequestering/inhibition associated with mutant SOD1 protein aggregation, a variety of mechanisms have been suggested to explain how aggregates might be neurotoxic: Misfolded mutant SOD1 may associate with mitochondria, inhibiting their function (see section 1.1.7); high affinity of mutant, aggregated SOD1 for the dynein-dynactin complex could interfere with the axonal transport of other cargoes (Breuer et al., 1987, Collard et al., 1995, Williamson and Cleveland, 1999); misfolded mutant SOD1 could activate the unfolded protein response (UPR) by inducing endoplasmic reticulum (ER) stress (Atkin et al., 2006, Atkin et al., 2008).

1.1.6 Mitochondrial Abnormalities in Mouse Models of SOD1-Related fALS

Mitochondrial dysfunction is a key feature of multiple neurodegenerative diseases, including ALS (reviewed in Lin and Beal, 2006). Mouse models of fALS due to mutations in SOD1 demonstrate the importance of mitochondrial abnormalities in motor neuron death and disease pathogenesis. These abnormalities include: rounding/swelling and vacuolization of mitochondria, especially in the neurites (SOD1G93A and SOD1G37R transgenic mice, though not observed in a SOD1G85R model; Dal Canto, 1994, Wong et al., 1995, Bruijn et al., 1997, Bendotti et al., 2001), reduced respiratory chain enzyme activity of complex I ($SOD1^{G93A}$; Browne et al., 1998), increased production of toxic reactive oxygen species (ROS; $SOD1^{G93A}$ model; Muller et al., 2007), reduced mitochondrial membrane potential ($SOD1^{G93A}$ and $SOD1^{G85R}$; Nguyen et al., 2009), reduced calcium buffering capacity ($SOD1^{G93A}$; Damiano et al., 2006), abnormal aggregation and axonal transport of mitochondria (in motor neurons derived from $SOD1^{G93A}$ mice; De Vos et al., 2007), oxidative damage to mitochondrial DNA ($SOD1^{G93A}$ mice; Warita et al., 2001), impairment of mitochondrial

DNA repair mechanisms ($SOD1^{G93A}$ mice; Murakami et al., 2007), and inappropriate associations of mutant SOD1 on and within the mitochondria ($SOD1^{G93A}$, ^{G85R}, ^{G37R}, and $^{H46R/H48Q}$ mice; Liu et al., 2004, Pasinelli et al., 2004, Vijayvergiya, 2005, Vande Velde, 2008, Israelson et al., 2010, Pedrini et al., 2010).

In the $SOD1^{G93A}$ transgenic mouse and culture models, mitochondrial abnormalities occur early in the disease process and before appearance of inclusions in motor neurons (Dal Canto, 1994, Kong and Xu, 1998, Tradewell et al., 2009). Therefore, mitochondrial dysfunction could be a promising target for developing interventions to prevent early pathogenic mechanisms leading to neuronal dysfunction and death.

1.1.7 Relationship Between Mutant SOD1 and Mitochondrial Abnormalities

In transgenic mouse models of ALS, mutant SOD1 accumulated and aggregated in the intermembrane space (IMS) and matrix (Vijayvergiya, 2005) of mitochondria to a greater extent than wild-type SOD1, particularly in the CNS (Liu et al., 2004, Ahtoniemi et al., 2008, Goldsteins et al., 2008). It has been suggested that accumulation of mutant SOD1 at the outer mitochondrial membrane interferes directly with the mitochondrial protein import machinery (Liu et al., 2004). In a neuroblastoma cell line model of fALS, localization of mutant SOD1 to the mitochondria led to increased ROS production and apoptotic cell death via a mitochondrial pathway, specifically, by inducing cytochrome-c release from the mitochondria, followed by activation of caspase 9 and caspase 3 (Takeuchi et al., 2002, Soo et al., 2009). Another link between mutant SOD1 and the mitochondrial apoptotic pathway was the observation that mutant SOD1 binds to Bcl-2 in spinal cord mitochondria, inhibiting its anti-apoptotic function (Pasinelli et al., 2004). Finally, misfolded mutant SOD1 was shown to bind to the voltage-dependent anion

channel, VDAC1, located in the mitochondrial outer membrane, inhibiting its conductance of potassium and chloride ions, and reducing ADP transport across the mitochondrial outer membrane. Furthermore, this same study demonstrated that inhibition of VDAC1 was enough to speed the disease onset of $SOD1^{G37R}$ transgenic mice (Israelson et al., 2010).

Given the detrimental effects of mutant SOD1 on mitochondria, it would be desirable to find a method of preventing or ameliorating inappropriate interactions between them. One such putative method could involve improving the protein quality control mechanisms of the mitochondria, for example, by increasing the expression of mitochondrial heat shock proteins.

1.2 Heat Shock Proteins

1.2.1 Normal Function in the Central Nervous System and Relevance to ALS

The ubiquitously-expressed, evolutionarily-conserved heat shock proteins (Hsp), proteins with chaperoning activity, serve a variety of important functions in the nervous system, and are involved in protein transport, cell growth and differentiation, signal transduction, protein quality control, and prevention of apoptosis (Jolly and Morimoto, 2000, Nollen and Morimoto, 2002). A vital function of Hsps, with relevance to neurodegenerative diseases like ALS, in which protein misfolding is a key feature, is that they recognize misfolded proteins and either help re-fold them, or target them for degradation by the ubiquitin-proteasome system (Nollen and Morimoto, 2002). While many of the major heat shock proteins are primarily cytosolic, the mitochondria have their own protein quality control machinery made up of a complex variety of chaperones

and proteases, including the mitochondrial heat shock proteins Hsp60, Hsp10, and mtHsp70 (Voos and Rottgers, 2002, Chacinska et al., 2009, Tatsuta, 2009)

Unfortunately, stress response systems are compromised in ALS. In lumbar spinal cord of $SOD1^{G93A}$ or $SOD1^{G85R}$ transgenic mice, the activity of protein chaperones was shown to be reduced compared to clinically unaffected tissues (Bruening et al., 1999, Tummala et al., 2005). Indeed, mutant SOD1 itself may sequester or inhibit Hsps (Shinder et al., 2001, Matsumoto et al., 2006). Also relevant to ALS and the effectiveness of the heat shock response in the disease is that motor neurons specifically have a high threshold for Hsp induction compared to glia, and could be less able to defend themselves against protein misfolding, contributing to their vulnerability in ALS (Batulan et al., 2003).

1.2.2 Heat Shock Proteins as a Therapeutic Target in ALS

Up-regulation of Hsps in the nervous system is protective against stresses related to heat shock, ischemia and neurodegenerative disease (reviewed in Brown, 2007). Treatments that increase the heat shock response in the CNS have shown some promise as potential ALS therapies.

Increasing the amount of stress-inducible Hsp70 by gene transfer reduced formation of inclusions in cultured motor neurons expressing $SOD1^{G93A}$ and prolonged their viability (Bruening et al., 1999). Likewise, expression of a constitutively active form of the heat shock transcription factor, Hsf1, or treatment with the chemical Hsp90 inhibitor, geldanamycin was protective. Both treatments inducing the expression of Hsp40 and Hsp70 in motor neurons of primary dissociated spinal cord-DRG cultures

prepared from embryonic mice; these treatments prolonged viability of motor neurons in these cultures expressing $SOD1^{G93A}$ and reduced mutant SOD1 inclusion formation (Batulan et al., 2006).

The intraperitineal injection of recombinant human Hsp70 into $SOD1^{G93A}$ transgenic mice delayed disease onset, improved motor function, and increased lifespan. However, it was noted that injected Hsp70 was mainly detected in skeletal muscle, not in CNS tissues, and therefore the mechanism of neuroprotection was unclear (Gifondorwa et al., 2007).

Chemical inducers of Hsps have had some success in ameliorating pathogenesis in the $SOD1^{G93A}$ ALS mouse model. For example, celastrol treatment upregulated Hsp70 and prolonged lifespan in a dose-dependent manner (up to 13%), while preserving 30% more neurons in the lumbar spinal cord compared to untreated mice (Kiaei et al., 2005). Arimoclomol, a co-inducer of Hsps, improved limb function and motor neuron survival, and increased lifespan by 22% (Kieran et al., 2004). However, late-stage treatment was unable to preserve normal lifespan, despite improved motor function and reduced mutant SOD1 aggregation (Kalmar et al., 2008).

ALS interventions that boost the heat shock response have not addressed the possibility of up-regulating mitochondrial heat shock proteins. Given that mitochondrial dysfunction is an early-stage mechanism leading to motor neuron death, there might be benefits inherent in protecting mitochondria from the direct and indirect effects of protein misfolding or downstream effects, perhaps by increasing the expression of mitochondrial Hsps. The current project was inspired by this notion, along with results from a study on aging *Drosophila melanogaster*, in which increased expression of mitochondrial Hsp22

was neuroprotective, increasing lifespan by 30%, preserving motor neurons and locomotion, and increasing resistance to paraquat-induced oxidative stress and heat stress (Morrow et al., 2004). The mitochondrial heat shock protein Hsp60, the most well-studied mitochondrial Hsp, was chosen as a potential therapeutic target to study in our primary culture model of ALS.

1.3 Mitochondrial Heat Shock Protein Hsp60

1.3.1 Structure and Function

Hsp60 is a chaperone protein whose primary function is the folding and assembly of polypeptide chains imported into the mitochondrial matrix; thus, it is highly important in mitochondrial protein quality control, especially under conditions of cellular stress (Martin et al., 1992). Hsp60 is encoded in the nucleus by the *Hspd1* gene and bears a 26 amino acid N-terminal mitochondrial localization sequence. The N-terminus is cleaved to form mature Hsp60, which is mainly localized to the mitochondrial matrix and outer membrane (Singh et al., 1990, Soltys and Gupta, 1996). 15-20% of Hsp60 is extra-mitochondrial, distributed among the cytosol, plasma membrane, peroxisomes, and ER (Soltys and Gupta, 1996, Soltys and Gupta, 1999). Hsp60 is constitutively expressed in neuronal and non-neuronal tissues and up-regulated under conditions of cellular stress (Welch et al., 1993). Hsp60 folding activity is ATP-dependent (Martin et al., 1992), so can be impaired if mitochondria are damaged and unable to produce sufficient ATP, resulting in a positive feedback loop of continuous mitochondrial damage. Hsp10 is required to regulate the ATPase cycle for Hsp60-mediated folding of some proteins

(Frydman, 2001). This finding highlights the fact that ATP production and the mitochondrial heat shock response are interacting processes that contribute to mitochondrial function.

1.3.2 Mitochondrial Hsp60 in Neuroprotection

Hsp60 is of special interest in the study of the protection of the central nervous system, especially in the context of neurodegenerative disease and other cellular insults that cause neuronal death. Experimental evidence reveals ways in which Hsp60 is neuroprotective.

For example, over- expression of the bacterial Hsp60 homologue, GroEL, protected against cerebral ischemia and prevented the formation of ubiquitinated protein aggregates in a rat model of middle cerebral artery occlusion (Xu et al., 2006). In a gerbil model of transient ischemia, levels of Hsp60 increased in the CA1 region of the hippocampus, and were associated with delayed neuron death in this region. Furthermore, animals that were treated with recombinant adenovirus expressing Hsp60 after transient ischemia had more surviving CA1 pyramidal neurons than untreated controls (Hwang et al., 2007).

In a rat model of brain stem death induced by exposure to the pesticide mevinphos, Hsp60 exerted its neuroprotective effects by inhibiting the mitochondrial apoptotic cascade in the rostral ventrolateral medulla. Following pesticide exposure, Hsp60 synthesis was increased, and it formed complexes with mitochondrial and cytosolic Bax and mitochondrial Bcl-2, while dissociating from cytosolic Bcl-2, thus

blocking pro-apoptotic signaling. When Hsp60 function was inhibited using antisense oligonucleotides or anti-Hsp60 antiserum, apoptosis increased (Chan et al., 2007).

A study on the neuroprotective mechanisms of the dopamine precursor L-DOPA in the substantia nigra using a rat model of Parkinson's disease found that L-DOPA administration was associated with increased synthesis of Hsp70, Hsc70 (a heat shock cognate protein with properties similar to Hsp70), and mitochondrial Hsp60. Moreover, increased Hsp expression was associated with higher expression and activity of complex I of the mitochondrial respiratory chain. The authors hypothesized that increased levels of these Hsps is an early protective mechanism against neuronal damage caused by reactive oxygen and nitrogen species, known contributors to side-effects that limit the long-term effectiveness of L-DOPA treatment in Parkinson's patients (Calabrese et al., 2007). Unfortunately, the specific role of Hsp60 was not delineated in this study. Future work should be done to specify the role of each of the Hsps noted in this work.

Finally, in a primary cortical neuron culture model of Alzheimer's disease, over-expression of Hsp60 protected complex IV of the respiratory chain against β-amyloid stress and reduced the production of ROS, thus limiting apoptosis (Veereshwarayya et al., 2006).

1.3.3 Mitochondrial Hsp60 Mutations and Neurological Disease

To date, four different Hsp60 mutations have been found in families with neurodegenerative diseases. The $Hsp60^{D29G}$ mutation causes mitCHAP-60 disease, an autosomal recessive disorder involving brain hypomyelination and leukodystrophy (Magen et al., 2008). The $Hsp60^{Q461E}$ polymorphism was identified in one hereditary spastic paraplegia patient in a Danish cohort. *E. coli* expressing this polymorphism showed impaired growth across a variety of temperatures compared to wild-type Hsp60 ($Hsp60^{WT}$) (Hansen et al., 2007). $Hsp60^{V98I}$ and $Hsp60^{V72I}$ mutations have been linked to autosomal dominant hereditary spastic paraplegia type SPG13 (Hansen et al., 2002, Bross et al., 2008). *E. coli* transfected with plasmids expressing $Hsp60^{V98I}$ had impaired mitochondrial protein folding capacity, reduced ATPase activity, and limited cell growth across a range of temperatures compared to those expressing $Hsp60^{WT}$ (Bross et al., 2008). A similar growth impairment was observed using the $Hsp60^{V72I}$ mutation in the same *E. coli* system (Hansen et al., 2002).

In addition to the alleged disease-causing Hsp60 mutations described above, there is some evidence that Hsp60 polymorphism might act as a genetic modifier, lowering the age of onset of disease in patients harboring spastin mutation (Hewamadduma et al., 2008).

The $Hsp60^{V72I}$ mutation, the first Hsp60 mutation to be linked to hereditary spastic paraplegia SPG13, has been chosen as a protein of interest for the current study, which seeks to define the potential cytoprotective and cytotoxic effects of Hsp60 in cultured motor neurons.

1.4 Hereditary Spastic Paraplegia

1.4.1 Clinical Overview

Hereditary spastic paraplegia constitutes a heterogeneous group of genetic diseases causing axonal degeneration of motor neurons of the spinal cord, leading to progressive lower limb spasticity and weakness. Upper limbs are rarely involved, and bulbar symptoms are lacking (Donaghy, 1999). Age of onset ranges from childhood to the seventh decade of life, though adult onset is most common, with patients living normal lifespans. The estimated prevalence of hereditary spastic paraplegias is 3-10 in 100 000. Inheritance has been linked to at least 17 different genes, and subtypes of the disease may be dominant, recessive, or X-linked (Salinas et al., 2008). The majority of autosomal dominant cases are due to mutations in *SPAST* and *SPG3A*, which encode the proteins spastin and atlastin, account for 40% and 10% of afflicted families, respectively (Stevanin et al., 2008). As previously mentioned, a rare group of autosomal dominant hereditary spastic paraplegia has been associated with mutations in *Hspd1,* the gene encoding Hsp60 (Hansen et al., 2002, Hansen et al., 2007, Bross et al., 2008). However, evidence for mutation in Hsp60 as the cause of disease and studies explaining the underlying disease mechanisms are scarce, with the Hsp60^{Q461E}, ^{V98I}, and ^{V72I} mutations having been identified in just one hereditary spastic paraplegia pedigree each.

1.4.2 Mitochondrial Abnormalities in Hereditary Spastic Paraplegia

Just as mitochondrial dysfunction is an important pathogenic mechanism in ALS and other neurodegenerative diseases, so too does it likely contribute to motor neuron death in some subtypes of hereditary spastic paraplegia. Because Hsp60 is a major mitochondrial protein chaperone mutated in some forms of hereditary spastic paraplegia,

it is likely that cell death resulting from Hsp60 mutations occurs via a mitochondrial pathway.

Specific mitochondrial abnormalities have already been found in an autosomal recessive subtype of hereditary spastic paraplegia, SPG7, which is caused by mutations in paraplegin, a metalloprotease subunit of the inner mitochondrial membrane m-AAA protease complex, whose normal function is in the turnover of misfolded respiratory chain peptides and in ribosome assembly in the mitochondria (Arnold and Langer, 2002, Nolden et al., 2005).

Muscle biopsies of patients with this disease subtype showed cytochrome oxidase negative fibres and heavy succinate dehydrogenase staining, indicating a paucity of mitochondrial oxidative phosphorylation and failed compensation through excessive proliferation of mitochondria (Casari et al., 1998). In another study, biochemical analysis of respiratory chain enzyme activity of muscle biopsies from SPG7 patients revealed reduced complex I activity in five out of six patients. The enzyme activities of complex II, III, and IV in these patients were normal (Piemonte et al., 2001). In addition to impaired complex I activity, fibroblasts from SPG7 patients showed increased vulnerability to oxidative stress compared to controls, effects that could be reversed by transfecting the cells with paraplegin (Atorino et al., 2003).

In an animal model of SPG7, paraplegin null mice have abnormal, hypertrophic mitochondria, which accumulate in presynaptic terminals due to anterograde and retrograde axonal transport deficits. The mitochondrial abnormalities are seen prior to the observed distal axonopathy of spinal and peripheral motor neurons characteristic of SPG7 disease in these mice and in human patients (Ferreirinha et al., 2004).

Defective anterograde and retrograde axonal transport of mitochondria may be a common feature of multiple types of hereditary spastic paraplegia, as it was also observed

in a mouse model of spastin mutation (SPG4) (Kasher et al., 2009). However, not all mitochondrial abnormalities necessarily manifest as common pathways in all types of hereditary spastic paraplegia. One study examined mitochondrial dysfunction in muscle biopsies from patients with known spastin mutation (SPG4), or with SPG4/7 mutations excluded. Complex I and IV activities were significantly reduced in the non- SPG4/7 group, whereas respiratory chain enzyme activity was not reduced in the SPG4 group (McDermott et al., 2003). Unfortunately, the genetic causes of disease in the non-SPG4/7 group were not classified, limiting the interpretation of the results. It would be interesting to know if patients with a mutation in Hsp60 were included in this group, for example.

It is possible that mutations in *Hspd1* that lead to compromised Hsp60 function, such as the $Hsp60^{V72I}$ mutation, cause mitochondrial abnormalities as well, but the nature of these abnormalities has yet to be elucidated. A study of mitochondrial function in cultured skin fibroblast cells from a single SPG13 patient with the $Hsp60^{V98I}$ mutation did not detect significant alterations in mitochondrial membrane potential, sensitivity to oxidative stress, or cell viability compared to controls. However, the mRNA expression levels of genes encoding the mitochondrial matrix proteases Lon and ClpP were reduced in SPG13 patients' cells. The authors suggested a compensatory mechanism whereby the degradation of misfolded proteins in the mitochondria is slowed, allowing mutant Hsp60 more attempts to re-fold these substrates (Hansen et al., 2008). However, this hypothesis has yet to be tested. Overall, the evidence describing potential mitochondrial defects in SPG13 is scarce, and the literature reveals a lack of disease-relevant experimental systems in which to investigate them, especially systems more applicable to the study of mitochondrial involvement in the motor neuron pathology and axonal degradation that are paramount in the disease. The primary dissociated spinal cord culture model of fALS due to SOD1 mutation is an experimental system that could be modified to study

potential mitochondrial involvement in hereditary spastic paraplegia SPG13. The current study uses a modified version of the fALS culture model, expressing $Hsp60^{V72I}$ in cultured motor neurons instead of $SOD1^{G93A}$, and employing experimental techniques already established for studying mitochondria in that model.

CHAPTER 2 –Rationale and Hypotheses

2.1 Rationale and Experimental Approach

In a model of aging in *Drosophila*, the over-expression of the mitochondrial heat shock protein Hsp22 in motor neurons increased lifespan, prolonged neuronal viability and improved motor activity during oxidative stress induced by paraquat (Morrow et al., 2004). Therefore, it is possible that over-expression of mammalian mitochondrial heat shock proteins, such as Hsp60, might have similar protective effects on motor neurons in a model of ALS.

On the other hand, the disruption of normal Hsp60 function is linked to neurological disease, and yet the specific involvement of mitochondrial dysfunction in Hsp60-related neurodegeneration has yet to be adequately described.

Mitochondria seem to be a common link for explaining part of the pathogenic mechanisms in a variety of neurodegenerative diseases, and therefore the study of mitochondrial dysfunction has broad relevance to the elucidation of neurodegenerative pathology.

For all experiments described, a primary dissociated spinal cord culture model was used to test the hypotheses. Plasmids that encode disease-causing proteins or their wild-type counterparts were introduced into motor neurons by intranuclear microinjection (this will be described in more detail in the Materials and Methods section). Previous work has shown that when $SOD1^{G93A}$ is expressed in this manner, subsequent motor neuron pathology mirrors that found in the human disease, compared to $SOD1^{WT}$, which does not manifest a disease phenotype, and is used as a control for overexpression of protein (Durham et al., 1997). $SOD1^{G93A}$-expressing motor neurons develop SOD1 inclusions by

day 3 post-microinjection. They also die earlier and at a faster rate than their $SOD1^{WT}$-expressing counterparts over 7 days of viability monitoring. Furthermore, defects in calcium handling and abnormal mitochondrial morphology and function are observed in this culture model as early pathological events (as early as day 1 and day 2 post-microinjection, respectively), preceding the formation of SOD1 inclusions and reduction in viability of mutant SOD1-expressing motor neurons (Tradewell et al., 2009, Tradewell et al., 2011). Therefore, this model constitutes a tractable system in which to test whether a mitochondrial Hsp, Hsp60, mitigates the effect of mutant SOD1 on mitochondria and neuronal viability. It also lends itself to modeling other neurodegenerative diseases, through the expression of plasmids that encode for other disease-causing proteins, such as the $Hsp60^{V72I}$ protein that causes hereditary spastic paraplegia SPG13.

2.2 Hypothesis 1

Increasing Hsp60 in motor neurons expressing mutant SOD1 reduces mitochondrial abnormalities, prolongs viability and ameliorates other signs of toxicity including formation of inclusions.

2.2.1 Specific Research Aims of Hypothesis 1

Objective: to determine if increasing Hsp60 expression is protective in a primary culture model of fALS:

1. By prolonging viability of motor neurons expressing mutant SOD1
2. By reducing formation of mutant SOD1 inclusions and/or preventing mitochondrial rounding caused by mutant SOD1

2.3 Hypothesis 2

Expression of Hsp60^{V72I}, a mutant Hsp60 linked to hereditary spastic paraplegia SPG13, induces mitochondrial abnormalities in cultured motor neurons, modeling the disease.

2.3.1 Specific Research Aims of Hypothesis 2

Objective: to model hereditary spastic paraplegia 13 by introducing Hsp60^{V72I} into motor neurons in primary dissociated spinal cord-DRG culture

1. By comparing the viability of motor neurons expressing Hsp60^{V72I} vs. Hsp60^{WT}

2. By comparing mitochondrial morphology and function in motor neurons expressing Hsp60^{V72I} vs. Hsp60^{WT} according to the following measures:

- Mitochondrial morphology (length)
- Mitochondrial membrane potential ($\Delta\psi$)
- Resistance to oxidative stress
- Axonal transport of mitochondria

3. In the event of negative results, to demonstrate that the endogenous murine Hsp60 does not mitigate/mask a disease-relevant phenotype potentially induced by the introduction of exogenous mutant Hsp60 plasmids in our cultures. This will be achieved by examining the effect of expressing the ATPase deficient mutant, Hsp60^{D423A}, in our primary cultures and measuring:

- Motor neuron viability
- Mitochondrial morphology (length)
- Mitochondrial membrane potential ($\Delta\psi$)

CHAPTER 3 –Materials and General Methods

3.1 Tissue Culture

Dissociated spinal cord-DRG cultures were prepared from embryonic day 13 (E13) CD1 mice (Charles River Laboratories, Wilmington, MA). Spinal cords were removed by dissection and placed in a 60 mm dish containing 1mL of dissection medium (5.5 mM dextrose, 58.4 mM sucrose, 6.8 mM NaCl, 0.27 mM KCl, 0.008 mM $Na_2HPO_4 \cdot 7H_2O$, 0.011 mM KH_2PO_4, 9.86 mM HEPES, pH 7.4). Cords were cut into pieces of approximately 1mm with a scalpel. To dissociate the tissue, an additional 2 mL of dissection medium containing 250 µL of 2.5% trypsin (Invitrogen Life Technologies, Burlington, ON) was added to the dish, which was then incubated for up to 30 min at 37°C. Trypsin activity was neutralized using 3 mL modified N3 medium (minimum essential medium containing 5 g/L glucose, 3% horse serum, 10 µg/mL bovine serum albumin, 26 ng/mL selenium, 20 µg/mL triiodothyronine, 10 µg/mL insulin, 200 µg/mL transferrin, 32 µg/mL putrescine, 9.1 ng/mL hydrocortisone, 13 ng/mL progesterone, and 10 ng/mL nerve growth factor). Further dissociation was achieved by light trituration with a pasteur pipette. Cells were plated at a density of 375 000 per well in 12-well Nunclon culture dishes containing round glass 18 mm coverslips, or at a density of 900 000 per well in 6-well Nunclon culture dishes containing 25mm round glass coverslips, coated with poly-D-lysine and Matrigel basement membrane matrix (Invitrogen Life Technologies, Burlington, ON). Cells were maintained in modified N3 medium at 37°C and 4% CO_2. After 4-6 days in culture, cells were treated with 1.4µg/mL cytosine-β-D-arabinoside to minimize growth of non-neuronal cells. Medium was refreshed twice per week by aspirating half of the medium in each well and replacing it with new medium. Cultures were utilized for experiments 3-8 weeks after dissociation. Motor neurons at this age *in vitro* are morphologically distinct from other cells in the cultures, having large cell bodies and tapered, branching dendrites containing neurofilaments that give the

neurites a fibrillar appearance. Furthermore, it has been previously established that motor neurons in these cultures express biological markers such as SM132 (Durham et al., 1997), choline acetyltransferase, (Durham, 1992), and Hb9, a motor neuron-specific transcription factor (Arber et al., 1999, Gingras et al., 2007).

3.2 Intranuclear Microinjection

Plasmid DNA was introduced into motor neurons by intranuclear microinjection. Standard transfection methods using liposome or calcium phosphate are ineffective in these cultures. While electroporation and viral transduction are more effective alternatives, these methods often result in a higher transfection rate in the other cell types, such as glial cells and DRG neurons, compared to motor neurons. The benefits of intranuclear microinjection are many: specific cells (motor neurons) can be targeted; multiple plasmids can be introduced simultaneously into each injected neuron, and the concentrations of these plasmids can be titrated in order to use the lowest amount necessary to achieve high-efficiency expression; finally, microinjection lends itself to live cell imaging of individual motor neurons injected with genes encoding fluorescent probes, while minimizing background fluorescence.

Microinjection needles were made by pulling glass capillaries (1.0 mm diameter, 3 inches long, with quick fill fibres; World Precision Instruments, Sarasota, FL) using a Narashige puller PN-3. Microinjection was carried out using an Eppendorf 5246 (or Eppendorf FemtoJet transjector) and an Eppendorf 5171 micromanipulator. To improve viscosity, all injectates contained 20mg/mL 70 kDa dextran, linked or unlinked to fluorescent isothiocyanate (FITC; Invitrogen Life Technologies, Burlington, ON), a low-toxicity fluorescent indicator of injected motor neurons. Coverslips with dissociated spinal cord cultures were transferred into 35 mm dishes containing minimum essential

medium (MEM; Invitrogen Life Technologies, Burlington, ON) without bicarbonate ($NaHCO_3$) or L-glutamine, supplemented with 5 g/L D-glucose and placed on the stage of a Zeiss Axiovert 35 microscope. Motor neurons were visualized using a 40x objective during microinjection. Following microinjection, each culture was transferred into a 35 mm dish containing 2mL of modified N3 medium with 0.75% gentamicin (Sigma Aldrich, Oakville, ON) to prevent bacterial growth. All experimental data was obtained at minimum 16 hours post-microinjection to ensure the exclusion of motor neurons damaged by the injection procedure and to allow for injected plasmid DNA to be expressed.

For each mutant protein expressed, the corresponding wild-type protein was expressed in parallel sets of cultures as a control. To control for any damaging affects to motor neurons caused by the microinjection procedure itself, empty vector conditions were also included.

3.3 Plasmid Expression Vectors

Each plasmid expression vector was titrated to find the lowest concentration at which protein expression could be detected above background levels by visualizing injected motor neurons using immunocytochemistry and epifluorescent microscopy. Human $SOD1^{WT}$ and $SOD1^{G93A}$ (mutant SOD1 with a glycine to alanine substitution at position 93) in pCEP4 plasmid were used at a concentration of 100-200 µg/mL. Human $SOD1^{WT}$ and $SOD1^{G93A}$ in pcDNA3 were used at a concentration of 40 µg/mL. Myc-tagged human $Hsp60^{WT}$ in pcDNA3.1 was a gift from Dr. Thomas Juhl Corydon (Institute of Human Genetics, University of Aarhus, Denmark) and was used at 10 µg/mL. Myc-tagged human $Hsp60^{V72I}$ in pcDNA3.1 (mutant Hsp60 with a valine to isoleucine substitution at position 72) and myc-tagged human $Hsp60^{D423A}$ in pcDNA3.1

(mutant Hsp60 with aspartic acid to alanine substitution at position 423) were made by Norclone Biotech Laboratories (London, Ontario, Canada) and used at 10 μg/mL. It should be noted that myc tags were used with the Hsp60-encoding plasmids in order to help distinguish human-Hsp60-injected cells from background cells, which express high levels of endogenous mouse Hsp60. pOCTeGFP was supplied by Dr. Heidi McBride (University of Ottawa, Ontario, Canada) and used at 1-2.5 μg/mL. eGFP was obtained from CloneTech and used at a concentration of 5-10 μg/mL. All plasmids were introduced into motor neurons by intranuclear microinjection as previously described.

Typically at least 20 viable motor neurons per culture contained the specified fluorescent indicator on day one post-microinjection. Each experimental condition was run in triplicate cultures from the same culture batch, and each experiment was repeated in at least two separate culture batches to verify reproducibility of results.

3.4 Immunocytochemistry

Cultures were transferred into 35mm dishes containing approximately 2 mL of 3% paraformaldehyde dissolved in phosphate-buffered saline (PF-PBS) and fixed for 10 minutes. PF-PBS was aspirated and replaced with 2 mL of 0.5% Nonidet P-40 PBS (NP-40-PBS) and cultures were permeabilized for 1 minute. NP-40-PBS was aspirated and 2 mL of 3% PF-PBS was added to the dish to fix cultures for an additional 2 minutes. PF-PBS was then aspirated and replaced with 2 mL of 5% horse serum-PBS (HS-PBS) and the cultures blocked for 30 minutes at room temperature to prevent non-specific binding of antibodies. All primary and secondary antibodies were diluted in 5% HS-PBS. Cultures were incubated in primary antibodies for one hour at room temperature. Cultures were then washed twice briefly in PBS, followed by 3 washes of 3 minutes each in PBS. Secondary antibodies were applied for 30 minutes to one hour at room

temperature. Cultures were again washed in PBS as above. Cultures were mounted on glass microscopy slides using ImmuMount (Fisher Scientific, Ottawa, ON) and allowed to dry before imaging.

Primary antibodies: mouse anti-SOD1 (1:300, Sigma-Aldrich, Oakville, ON, clone #SD-G6), goat anti-c-MYC (1:600, Santa Cruz Biotechnologies, Santa Cruz, CA, A-14-G), rabbit anti-Hsp60 (1:100, Santa Cruz Biotechnologies, Santa Cruz, CA, H-300), mouse anti-cytochrome-c (1:400, BD Pharmingen, Mississauge, ON).

Secondary antibodies used for immunocytochemistry: donkey anti-mouse Cy3 (1:300, Jackson ImmunoResearch, catalogue # 16160084), donkey anti-goat Cy3 (1:300, Jackson ImmunoResearch, catalogue # 705165147), donkey anti-goat Cy5 (1:300, Jackson ImmunoResearch, catalogue # 705175003), donkey anti-rabbit Cy3 (1:300, Jackson ImmunoResearch, catalogue #711165152), donkey anti-mouse Cy2 (1:300, Jackson ImmunoResearch, catalogue #715225151).

3.5 Imaging

Where epifluorescent microscopy is indicated, motor neurons were imaged using a Zeiss Axiovert 35 inverted microscope equipped with a Hamamatsu Orca camera, and Sutter Lambda 10 and 10C filter wheels, for excitation and emission, respectively. Filters were obtained from Chroma (Chroma Technologies, Rockingham, VT). MetaFluor 6.05 software (Molecular Devices, Downingtown, PA) was used for image acquisition.

Where confocal microscopy is indicated, cells were imaged using a LSM510 Meta system and Zeiss Axiovert 200 microscope. Images were acquired using a 40X 1.4 NA or 63X 1.3 NA objective and LSM Image examiner software.

Image analyses were performed using NIH ImageJ software (http://rsb.info.nih.gov/ij/).

3.6 Determination of Motor Neuron Viability

Plasmid expression vectors were introduced into motor neurons by intranuclear microinjection, as previously described. All of the injectates contained 20mg/mL 70 kDa dextran-FITC as an inert fluorescent indicator of injected cells.

To assess viability, live motor neurons containing the indicator were counted using epifluorescent optics daily for 7 days, beginning 16-24 hours post-microinjection (day 1). Each motor neuron also was viewed under transmitted light to determine viability. Motor neurons were considered viable if the dextran-FITC was present and distributed diffusely through the nucleus and the neurons were phase-bright, with intact cell bodies and nuclei and dark nucleoli.

Following viability assessment in live motor neurons on day 7-post microinjection, cultures were were fixed and immunocytochemistry was performed to verify the co-expression of human SOD1 and Hsp60.

3.7 Assessment of SOD1 Inclusion Formation

To determine whether expression of human $Hsp60^{WT}$ could ameliorate inclusion formation in motor neurons expressing $SOD1^{G93A}$, plasmids encoding $SOD1^{WT}$ or $SOD1^{G93A}$, with or without $Hsp60^{WT}$ were introduced into motor neurons by intranuclear microinjection as previously described.

Viability of motor neurons was assessed by epifluorescent microscopy daily throughout the experiment, as described in section 3.6, using a plasmid encoding pOCTeGFP as a fluorescent indicator of injected motor neurons. pOCTeGFP was used

to identify injected neurons instead of dextran-FITC as the fluorescent indicator in this experiment because it has a mitochondrial localization sequence, making it possible to examine mitochondrial morphology (see section 3.8) and SOD1 inclusion formation simultaneously in injected motor neurons.

On day 3 post-microinjection cultures were fixed and examined using immunocytochemistry and epifluorescent microscopy for expression of human SOD1 and Hsp60. It has been previously established that motor neurons expressing G93ASOD1 in our model begin showing inclusions at this time point (Durham et al., 1997). The SOD1 antibody being used was chosen because of its ability to detect both diffuse and aggregated mutant SOD1, but does not label endogenous murine SOD1. Motor neurons were classified as having mutant SOD1 inclusions if the antibody detected small, round inclusion bodies in the cell soma, dendrites and axon.

3.8 Assessment of Mitochondrial Morphology

In order to visualize mitochondria and assess their morphology, plasmids encoding the mitochondrially-targeted eGFP, pOCTeGFP, along with the other proteins of interest, were introduced into motor neurons by intranuclear microinjection as previously described. Mitochondria in each motor neuron were viewed using epifluorescent microscopy and classified as having rounded (punctate) morphology, or reticular morphology, based on the appearance of the majority of mitochondria distinguishable in the cell body and processes.

For cases in which no obvious mitochondrial rounding occurred, mitochondrial lengths were measured in order to detect more subtle morphological changes. Images of live axonal mitochondria were obtained using epifluorescent microscopy and MetaFluor software. Axonal mitochondria were chosen because the linear organization of

mitochondria along the axon makes them easily distinguishable from one another. These images were then used to obtain mitochondrial length measurements using ImageJ software.

For all experiments, cultures were fixed and the expression of exogenous proteins was verified using immunocytochemistry.

3.9 Measurement of Mitochondrial Membrane Potential (ΔΨ)

Mitochondrial membrane potential (ΔΨ) was measured using tetramethyl rhodamine methyl ester (TMRM), a membrane-permeable, mitochondrially-targeted, potentiometric dye, whose uptake is directly proportional to ΔΨ (Buckman and Reynolds, 2001).

Plasmid expression vectors were introduced into motor neurons by intranuclear microinjection as previously described. All injectates contained a plasmid encoding for eGFP as a fluorescent indicator of injected cells.

On days 3 and 5 post-microinjection cultures were incubated in 100 nM TMRM dissolved in Eagle's Minimum Essential Medium (EMEM) for 30 minutes at 37˚C. Cultures were transferred to a live cell imaging chamber containing 10nM TMRM at 37˚C. TMRM was imaged using dsRED/Cy3/Texas Red filters. All image-capture parameters (for example, exposure time, binning and gain settings) were kept constant to allow for comparison of fluorescence intensity values from different motor neurons. Intensity of TMRM fluorescence (pixel intensity) was measured using the trace function in MetaFluor by tracing around several individual mitochondria in neuronal processes where the cell is thin, to minimize the confounding influence of cell thickness.

As a control, the uncoupling agent FCCP was added to some TMRM-treated cultures to show that a loss of $\Delta\Psi$ would, in fact, lead to a loss of TMRM from the mitochondria.

Following live imaging, all cultures were fixed and the expression of exogenous proteins was verified using immunocytochemistry.

3.10 Assessment of Resistance to Oxidative Stress

The superoxide generator paraquat dichloride was used as an oxidative insult to motor neurons in order to test their resistance to oxidative stress.

Plasmid expression vectors were introduced into motor neurons by intranuclear microinjection as previously described. All of the injectates contained a plasmid encoding eGFP as a marker of injected cells.

Motor neuron viability was assessed daily for 3 days post-microinjection as previously described, before the oxidative stressor was introduced, in order to establish a baseline of motor neuron health.

On day 3 post-microinjection cultures were treated with 5uM paraquat dichloride. This concentration of paraquat dichloride was chosen based on a titration experiment to determine a concentration that would achieve 50% viability reduction in the empty vector condition at 48 hours of treatment. The number of viable motor neurons in each culture was counted at 12, 24, 36, and 48 hours of paraquat treatment.

Following live imaging, all cultures were fixed and the expression of exogenous proteins was verified using immunocytochemistry.

3.11 Measurement of Mitochondrial Axonal Transport

The axonal transport of mitochondria was measured in live motor neurons using time-course imaging and kymograph analysis. In order to visualize mitochondria in live cultured motor neurons, plasmids encoding pOCTeGFP, plus the protein of interest, were introduced by intranuclear microinjection, as previously described.

On days 3 and 5 post-microinjection, cultures were transferred to a live imaging chamber (Harvard Apparatus series 40 high profile microincubation chamber, model RC-40HP) containing 37˚C EMEM and placed on the stage of a Zeiss Axiovert 35 microscope. The axons of injected motor neurons were imaged using the time-course setting in MetaFluor, capturing one image every 4 seconds, times one-hundred images per axon. These time settings allowed for mitochondrial movement along the axon to be observed when images were collated to create movies using MetaFluor. For more detailed analysis, ImageJ was used to create kymographs (distance vs. time plots) of mitochondrial movement, from which the number of stationary vs. moving mitochondria could be counted for each axon; vertical lines on the kymographs indicated that no change in distance occurred over time, and therefore represented stationary mitochondria. Angled lines on the kymographs indicated change in distance over time, and therefore represented moving mitochondria.

Following live imaging, coverslips were fixed and permeabilized and exogenous protein expression was verified using immunocytochemistry, as previously described.

3.12 Statistical Analysis

Statistical analyses were performed using SPSS 16.0. Viability and oxidative stress data were analysed using the Wilcoxon log-rank test (life tables analysis). Mitochondrial rounding and inclusion formation data were each analysed using the Pearson chi-square. Mitochondrial measurements, axonal transport, and mitochondrial membrane potential data were analysed using one-way ANOVA or two-tailed t-test, where appropriate. $P<0.05$ was considered significant.

CHAPTER 4 – Results of Experiments Testing Hypothesis 1

Hypothesis 1: *Increasing Hsp60 in motor neurons expressing mutant SOD1 reduces mitochondrial abnormalities, prolongs viability and ameliorates other signs of toxicity including formation of inclusions.*

This hypothesis was tested by determining if increasing Hsp60 expression was protective in the culture model of fALS1; using the following measures of toxicity: viability of motor neurons expressing the ALS-causing mutant $SOD1^{G93A}$; formation of mutant SOD1 inclusions, and mitochondrial rounding.

4.1 Expression of Hsp60 did not prolong the viability of motor neurons expressing mutant Cu/Zn superoxide dismutase ($SOD1^{G93A}$)

The expression of $SOD1^{G93A}$ has been shown to reduce the viability of cultured motor neurons compared to $SOD1^{WT}$. The viability of motor neurons expressing $SOD1^{WT}$ or $SOD1^{G93A}$, with or without co-expression of $Hsp60^{WT}$, was assessed over a period of 7 days following microinjection of plasmid expression vectors.

The following combinations of plasmids were expressed:

$SOD1^{WT}$ in pCEP4 + pcDNA3 empty vector

$SOD1^{WT}$ in pCEP4 + $Hsp60^{WT}$ in pcDNA3.1

$SOD1^{G93A}$ in pCEP4 + pcDNA3 empty vector

$SOD1^{G93A}$ in pCEP4 + $Hsp60^{WT}$ in pcDNA3.1

As expected, the expression of $SOD1^{G93A}$ reduced the viability of motor neurons compared to expression of $SOD1^{WT}$ (**$P<0.01$, Wilcoxon log-rank test). However, the co-expression of human $Hsp60^{WT}$ did not significantly improve the viability of motor

neurons expressing mutant SOD1 (P=0.2808) (Figure 1). At least three cultures were injected per condition, with 24-44 motor neurons present per culture on day 1.

4.2 Expression of Hsp60 did not decrease the proportion of motor neurons with mutant Cu/Zn superoxide dismutase ($SOD1^{G93A}$) inclusions

As mutant SOD1 inclusion formation is a hallmark of pathogenesis in ALS patients, animal models and culture models, the ability of exogenous Hsp60 expression to reduce inclusion formation in cultured motor neurons expressing mutant SOD1 was assessed.

The following plasmid combinations were introduced into motor neurons by intranuclear microinjection:

$SOD1^{WT}$ in pCEP4 + pcDNA3 empty vector + pOCTeGFP

$SOD1^{WT}$ in pCEP4 + $Hsp60^{WT}$ in pcDNA3.1+ pOCTeGFP

$SOD1^{G93A}$ in pCEP4 + pcDNA3 empty vector + pOCTeGFP

$SOD1^{G93A}$ in pCEP4 + $Hsp60^{WT}$ in pcDNA3.1+ pOCTeGFP

A significant proportion (36%) of motor neurons expressing $SOD1^{G93A}$ developed SOD1-positive inclusions, whereas those expressing $SOD1^{WT}$ had exclusively diffuse SOD1 expression (Figure 2a). The co-expression of $Hsp60^{WT}$ did not cause any inclusion formation in motor neurons expressing $SOD1^{WT}$, nor did it significantly reduce inclusion formation in motor neurons expressing $SOD1^{G93A}$ (P=0.1955, Pearson Chi-Square) (Figure 2b). Confocal microscopy revealed that $Hsp60^{WT}$ did not co-localize with mutant SOD1 inclusions, indicating that the inclusions were not mitochondrial, and that $Hsp60^{WT}$ was neither recruited to, nor sequestered by, these inclusions (Figure 2c). Each

experiment included at least three cultures per condition, with at least 20 motor neurons present on day 1. Data from three experiments were combined for statistical analysis.

4.3 Expression of Hsp60 did not decrease mitochondrial rounding in motor neurons expressing mutant Cu/Zn superoxide dismutase ($SOD1^{G93A}$)

Mitochondrial abnormalities, including morphological changes, occur early in the $SOD1^{G93A}$ primary culture model of fALS. Therefore, the mitochondrial morphology of motor neurons expressing $SOD1^{WT}$ or $SOD1^{G93A}$, with or without co-expression of $Hsp60^{WT}$, was compared. Plasmid combinations used for this experiment were the same as in section 4.2, as mutant SOD1 inclusion formation and mitochondrial morphology were assessed simultaneously in the same motor neurons.

The mitochondrially-targeted green fluorescent protein, pOCTeGFP, was used to visualize mitochondria in fixed motor neurons using epifluorescent microscopy. Obvious mitochondrial rounding occurred in a significant proportion (40%) of motor neurons expressing $SOD1^{G93A}$, as expected. There was no rounding of the mitochondria in motor neurons expressing $SOD1^{WT}$ (for representative images, see Figure 3a). Co-expression of $Hsp60^{WT}$ did not affect mitochondrial rounding, neither causing it in the $SOD1^{WT}$-expressing motor neurons, nor ameliorating it in the $SOD1^{G93A}$-expressing motor neurons ($P=0.1118$, Pearson Chi-Square) (Figure 3b).

CHAPTER 5 – Results of Experiments Testing Hypothesis 2

Hypothesis 2: *Expression of Hsp60^{V72I}, a mutant Hsp60 linked to hereditary spastic paraplegia SPG13, induces mitochondrial abnormalities in cultured motor neurons, modeling the disease.*

The objective was to model hereditary spastic paraplegia 13 by introducing Hsp60^{V72I} into motor neurons in primary dissociated spinal cord-DRG cultures and assessing any phenotype relative to Hsp60^{WT} according to the effects on neuronal viability, resistance to oxidative stress, mitochondrial morphology (length) and mitochondrial function ($\Delta\psi$ and axonal transport).

5.1 Expression of Hsp60^{V72I} did not reduce the viability of cultured motor neurons compared to Hsp60^{WT}

If the Hsp60^{V72I} mutation causes HSPG13, then a neuronal phenotype would be expected. To assess whether the expression of the HSPG13-associated mutant, Hsp60^{V72I} compromised the viability of cultured motor neurons, the following plasmids were introduced into motor neurons by intranuclear microinjection:

Hsp60^{WT} in pcDNA3.1

Hsp60^{V72I} in pcDNA3.1

pcDNA3.1 empty vector

and live, healthy motor neurons containing a fluorescent indicator were counted daily for 7 days. Though motor neurons expressing Hsp60^{V72I} had significantly reduced viability compared to those injected with empty vector ($P<0.01$), viability was not compromised compared to motor neurons expressing Hsp60^{WT} ($P=0.2764$) (Figure 4). Three cultures

were injected per condition, with 20-76 motor neurons present per culture on day 1, and results were verified in 4 different culture batches.

5.2 Expression of Hsp60^{V72I} did not cause mitochondrial rounding or shortening in cultured motor neurons

Mitochondrial rounding is a common feature of mitochondrial involvement in models of disease, including motor neuron pathology in the SOD1^{G93A} ALS primary culture model, mitochondrial morphology was assessed in cultured motor neurons expressing the HSPG13 mutant, Hsp60^{V72I}, to determine whether these disease models share common mitochondrial pathology.

For this experiment, the following plasmid combinations were used:

Hsp60^{WT} in pcDNA3.1 + pOCTeGFP

Hsp60^{V72I} in pcDNA3.1 + pOCTeGFP

pcDNA3.1 empty vector + pOCTeGFP

On day 3 or day 5 post-microinjection, mitochondria in the processes of live motor neurons were visualized by epifluorescent microscopy using the mitochondrially-targeted green fluorescent protein, pOCTeGFP, and imaged using MetaFluor. No obvious mitochondrial rounding was observed, with all images showing reticular networks of mitochondria (Figure 5a). Therefore, mitochondria were measured using ImageJ and lengths were averaged for each axon, and then average mitochondrial length per axon was averaged for multiple axons per condition, in order to detect more subtle changes in mitochondrial length. Day 3 data includes measurements from 268-365

mitochondria per condition (11-14 axons). Day 5 data includes measurements from 1206-2162 mitochondria per condition (51-73 axons). There was no significant difference in the length of mitochondria among injection conditions at either time point (day 3 P = 0.3235, day 5 P = 0.1980) (Figure 5b).

5.3 Expression of Hsp60^{V72I} did not reduce the mitochondrial membrane potential (ΔΨ) in cultured motor neurons

Reduced mitochondrial membrane potential (ΔΨ) is associated with mitochondrial damage and reduced oxidative phosphorylation capacity, and is a pathogenic feature in neurodegeneration. The membrane-permeable, potentiometric dye, tetramethyl rhodamine methyl ester (TMRM), whose uptake is proportional to ΔΨ (Buckman and Reynolds, 2001), was used to determine whether or not the expression of Hsp60^{V72I} in motor neurons leads to a reduction in ΔΨ.

The following plasmids were introduced into cultured motor neurons by intranuclear microinjection:

Hsp60^{WT} in pcDNA3.1 + eGFP

Hsp60^{V72I} in pcDNA3.1 + eGFP

pcDNA3.1 empty vector + eGFP

On day 3 or day 5 post-microinjection, each culture was treated with TMRM as described in materials and methods, and the TMRM signal in mitochondria of axons and dendrites of live motor neurons was visualized using dsRED/Cy3/Texas Red filters imaged using MetaFluor (Figure 6a). TMRM fluorescence intensity values from several individual mitochondria per neuronal process were obtained in MetaFluor and averaged for each neuron. As a control, the uncoupling agent FCCP was added to some TMRM-

treated cultures to show that a loss of ΔΨ would, in fact, lead to a loss of TMRM from the mitochondria. FCCP-induced loss of ΔΨ is shown in figure (6b), with eGFP marker shown to demonstrate that images are in focus. The TMRM signal, and therefore, ΔΨ, was not significantly lower in motor neurons expressing $HSP60^{V72I}$ compared to those expressing $HSP60^{WT}$ or empty vector (Figure 6c) (Day 3 P = 0.7340. Day 5 P =0.3369). Day 3 data includes measurements from 43-47 motor neurons per condition. Day 5 data includes measurements from 34-47 motor neurons per condition.

5.4 Expression of $Hsp60^{V72I}$ did not increase the vulnerability of cultured motor neurons to oxidative stress

Even in the absence of a phenotype with expression of a mutant protein, subjecting the cells to toxic stress might bring out a subthreshold effect. Oxidative stress is a common stress used for this purpose and resilience to stress might be reduced if mitochondrial function and other stress responses were compromised. The superoxide generator paraquat dichloride was used as an oxidative insult to test the relative oxidative stress resistance of motor neurons expressing $Hsp60^{V72I}$.

The following plasmids were introduced by intranuclear microinjection:

$Hsp60^{WT}$ in pcDNA3.1 + eGFP

$Hsp60^{V72I}$ in pcDNA3.1 + eGFP

pcDNA3.1 empty vector + eGFP

Injected motor neurons were identified by their eGFP signal using epifluorescence microscopy, and viability was assessed by viewing motor neurons under transmitted light as previously described. Viability was assessed daily for 3 days post-microinjection,

before the oxidative stressor was introduced. On day 3 post-microinjection cultures were treated with 5uM paraquat dichloride. Viable motor neurons continued to be counted at 12, 24, 36, and 48 hours of paraquat treatment. While paraquat treatment caused a general decline in motor neuron viability across all injection conditions, the viability of oxidatively-stressed motor neurons expressing $Hsp60^{V72I}$ was not significantly reduced compared to those injected with $Hsp60^{WT}$or empty vector (empty vector vs. $Hsp60^{WT}$ P=0.4476; empty vector vs. $Hsp60^{V72I}$ P=0.7999; $Hsp60^{WT}$ vs.$Hsp60^{V72I}$ P=0.3165) (Figure 7). Two to three cultures were injected per condition in two separate culture batches (for a total of 17 cultures), with 40-57 viable motor neurons per culture present on day 1. Exogenous protein expression was verified in all cultures by immunocytochemistry following the viability count at 48 hours of paraquat treatment.

5.5 Expression of $Hsp60^{V72I}$ did not impair the axonal transport of mitochondria in cultured motor neurons

In order for motor neurons to be healthy, they must be able to transport mitochondria to areas of the cell with high energy demand, for example to synapses. The axonal transport of mitochondria was measured in live motor neurons expressing $Hsp60^{V72I}$, $Hsp60^{WT}$, or empty vector, plus pOCTeGFP to visualize the mitochondria. On day 3 or day 5 post-microinjection, axons of live motor neurons were identified and, using the time-course setting in MetaFluor, imaged at a rate of 1 image every 4 seconds, for a total of 100 live images per axon. Images were collated to make time-course videos of mitochondrial movement in MetaFluor, and these videos were then used to create kymographs (distance vs. time plots) for the quantification of mitochondrial movement

(static vs. moving) using ImageJ. Representative kymographs are shown in Figure (8a). Stationary mitochondria are represented by vertical lines on the kymograph, while moving mitochondria are represented by diagonal lines. Following live imaging, each culture was fixed and permeabilized and exogenous protein expression was verified by immunocytochemistry. $Hsp60^{V72I}$ expression did not reduce the percentage of moving mitochondria in motor neuron axons compared to $Hsp60^{WT}$ or empty vector at either time point (Day 3 P=0.6355; Day 5 P =0.3538) (Figure 8b). Day 3 data includes 9-13 axons per condition. Day 5 data includes 13-27 axons per condition.

5.6 Expression of the ATPase-deficient mutant, $Hsp60^{D423A}$, did not reduce the viability, shorten the mitochondria, or reduce the mitochondrial membrane potential of cultured motor neurons compared to expression of $Hsp60^{V72I}$

Because negative results were obtained in all experiments with Hsp60V72I (viability, mitochondrial length, mitochondrial membrane potential, resistance to oxidative stress, axonal transport of mitochondria), it was necessary to demonstrate that the endogenous murine Hsp60 did not mitigate/mask a disease-relevant phenotype potentially induced by the introduction of exogenous mutant Hsp60 plasmids in our cultures. To address this, the ATPase deficient mutant, $Hsp60^{D423A}$, was expressed in our primary cultures and the following parameters were measured:

Motor neuron viability; Mitochondrial morphology (length);
Mitochondrial membrane potential ($\Delta\psi$)

It has previously been demonstrated that the $Hsp60^{D423A}$ mutant possesses about 2% of the ATPase activity of $Hsp60^{WT}$ (Bross et al., 2008), and can therefore be considered to have severely impaired chaperone activity.

$Hsp60^{D423A}$ or $Hsp60^{V72I}$, plus the appropriate fluorescent marker for each experiment, was expressed in cultured motor neurons and viability, mitochondrial length, and mitochondrial membrane potential were carried out as previously described. Exogenous protein expression was verified in all experimental cultures by immunocytochemistry.

The ATPase deficient $Hsp60^{D423A}$ caused no reduction in motor neuron viability compared to $Hsp60^{V72I}$ over 10 days of viability assessment (P=0.255; 2-3 cultures were injected per condition, with 21-38 motor neurons counted per culture on day 1) (Figure 9). Likewise, there was no difference in mitochondrial length (P=0.8497; 3 cultures injected per condition, 52-56 axons) (Figure 10), or in $\Delta\Psi$ (P=0.3980; 3 cultures injected per condition, 35-42 motor neurons per condition) (Figure 11) by day 5 post-microinjection.

FIGURES

Figure 1. Viability of motor neurons expressing wild-type or mutant Cu/Zn superoxide dismutase ($SOD1^{WT}$ or $SOD1^{G93A}$), with or without Hsp60. Motor neurons were injected with plasmids encoding $SOD1^{WT}$ or $SOD1^{G93A}$, with or without a plasmid encoding Hsp60, using dextran-fluorescein as a marker and counted for 7 days. Motor neurons were counted as viable if they appeared phase bright, had intact nuclei and dark nucleoli when viewed under transmitted light. A minimum of three cultures were injected per condition, with 24-44 motor neurons per culture present on day 1. Motor neurons expressing $SOD1^{G93A}$ had significantly reduced viability compared to those expressing $SOD1^{WT}$ (**$P<0.01$, Wilcoxon log-rank test). HSP60 failed to improve the viability of motor neurons expressing $SOD1^{G93A}$ ($P=0.2808$).

Figure 1

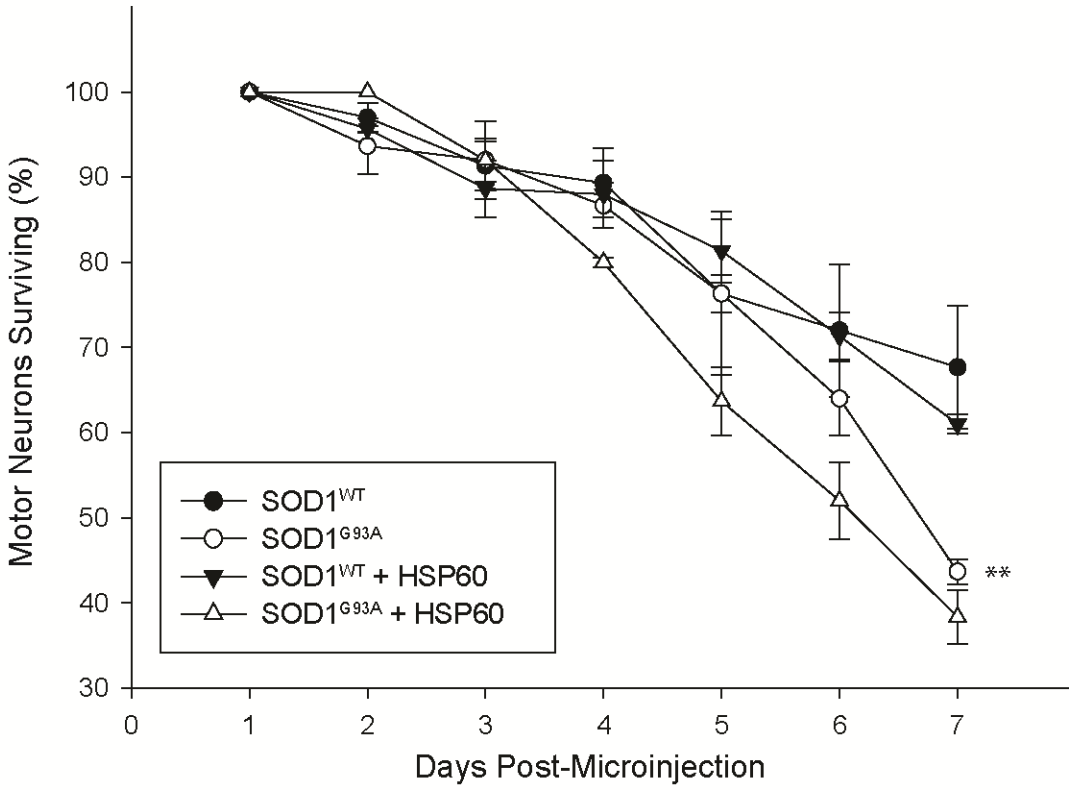

Motor Neurons Surviving (%)
100
90
80
70
60
50
40
30
0
1
2
3
4
5
6
7
Days Post-Microinjection
SOD1WT
SOD1G93A
SOD1WT + HSP60
SOD1G93A + HSP60
**

Figure 2. SOD1 inclusion formation in motor neurons expressing $SOD1^{WT}$ or $SOD1^{G93A}$, with or without Hsp60. Motor neurons were injected with plasmid encoding $SOD1^{WT}$ or $SOD1^{G93A}$ with or without a plasmid encoding Hsp60, using pOCTeGFP as a marker. On day 3 post-microinjection, cultures were subjected to immunocytochemistry using anti-SOD1 antibody, and the number of motor neurons with SOD1 inclusions was counted using epifluorescence microscopy. At least 3 cultures per condition with a minimum of 20 viable motor neurons per culture were used for each experiment. Data from three experiments are combined here, and expressed as mean % motor neurons with SOD1 inclusions, +/- SEM. SOD1 inclusions were present in some motor neurons expressing $SOD1^{G93A}$ but not in those expressing $SOD1^{WT}$. An example of $SOD1^{G93A}$ inclusion formation is compared to the diffuse expression of $SOD1^{WT}$ in **(a).** Hsp60 expression did not reduce the proportion of motor neurons with $SOD1^{G93A}$ inclusions **(b)** ($P=0.1955$, Pearson Chi-Square), nor did Hsp60 co-localize with these inclusions, showing that the inclusions were not mitochondrial **(c).**

Figure 2

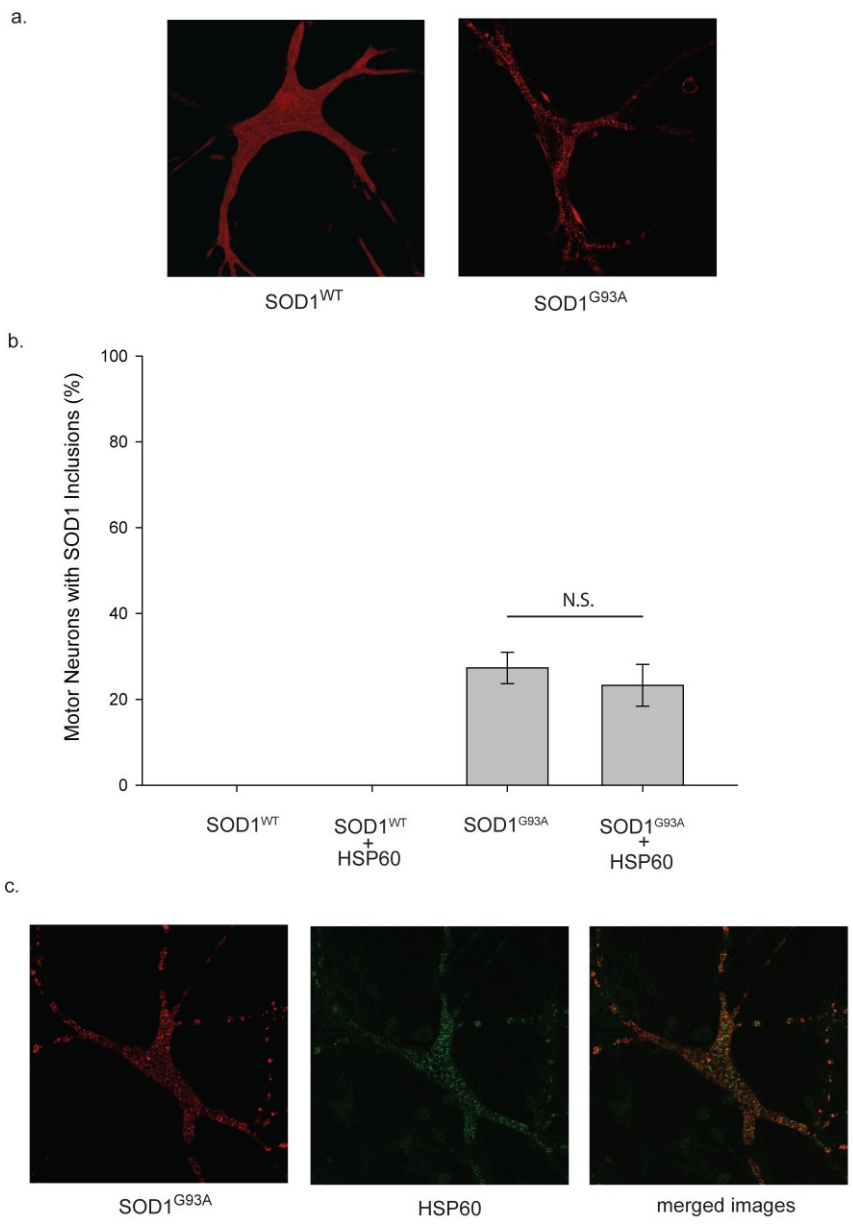
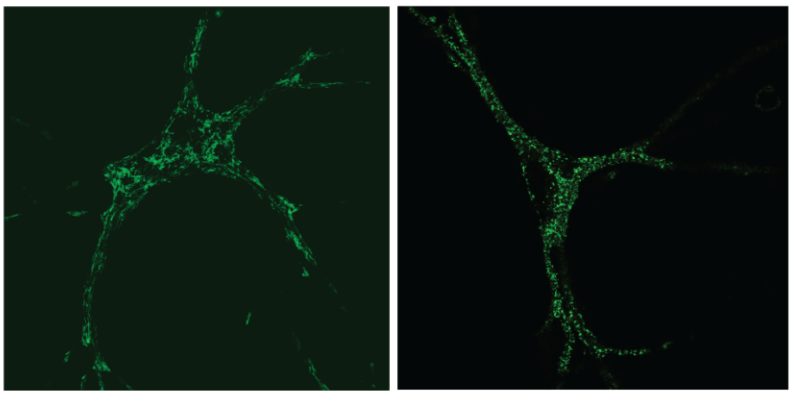

a.
SOD1WT
SOD1G93A
b.
Motor Neurons with SOD1 Inclusions (%)
100
80
60
40
20
0
N.S.
SOD1WT
SOD1WT + HSP60
SOD1G93A
SOD1G93A + HSP60
c.
SOD1G93A
HSP60
merged images

Figure 3. Mitochondrial rounding in motor neurons expressing $SOD1^{WT}$ or $SOD1^{G93A}$, with or without Hsp60. Motor neurons were injected with plasmid encoding $SOD1^{WT}$ or $SOD1^{G93A}$ with or without plasmid encoding Hsp60, using pOCTeGFP as a marker to visualize mitochondria. On day 3 post-microinjection, cultures were subjected to immunocytochemistry using anti-SOD1 antibody, and the number of motor neurons with rounded mitochondria was counted using epifluorescence microscopy. At least 3 cultures per condition with a minimum of 20 viable motor neurons per culture were used for each experiment. Data from three experiments are combined here and expressed at mean % of motor neurons with rounded mitochondria, +/- SEM. Mitochondrial rounding occurred in some motor neurons expressing $SOD1^{G93A}$, while those expressing $SOD1^{WT}$ maintained a reticular network of mitochondria **(a).** Hsp60 expression did not reduce the proportion of motor neurons with rounded mitochondria **(b)** (P=0.1118, Pearson Chi-Square).

Figure 3

a.


pOCTeGFP+SOD1WT
pOCTeGFP+SOD1G93A

b.

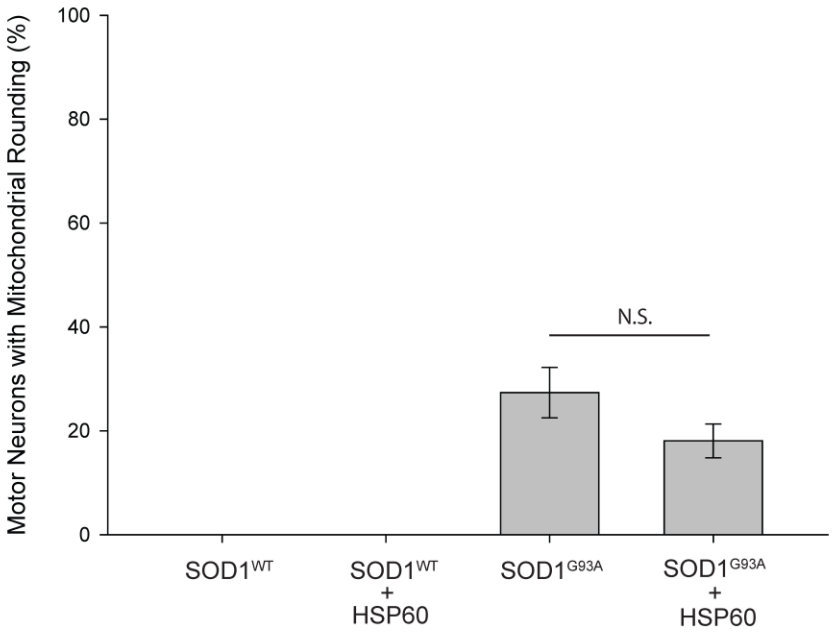

Motor Neurons with Mitochondrial Rounding (%)
100
80
60
40
20
0
N.S.
SOD1WT
SOD1WT + HSP60
SOD1G93A
SOD1G93A + HSP60

Figure 4. Viability of motor neurons expressing human $HSP60^{V72I}$ vs. $HSP60^{WT}$ vs. empty vector. Motor neurons were injected with plasmids encoding $HSP60^{WT}$, $HSP60^{V72I}$, or empty PCDNA3 vector, using dextran-fluorescein as a marker and counted for 7 days. Motor neurons expressing $HSP60^{V72I}$ had significantly reduced viability compared to those injected with empty vector (**P<0.01, Wilcoxon log-rank test), but not compared to those expressing $HSP60^{WT}$(P=0.2764). Three cultures were injected per condition in four separate culture batches (for a total of 36 cultures), with 20-76 motor neurons counted per culture on day 1.

Figure 4

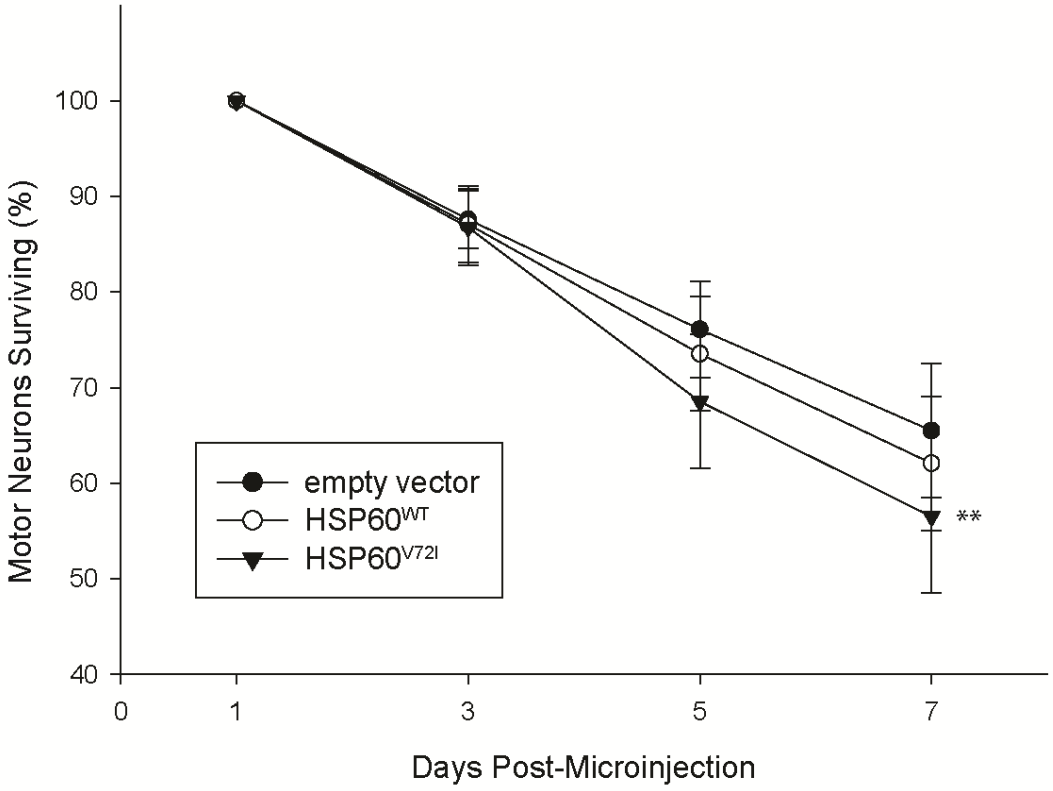

Motor Neurons Surviving (%)
100
90
80
70
60
50
40
0
1
3
5
7
Days Post-Microinjection
empty vector
HSP60WT
HSP60V72I
**

Figure 5. Mitochondrial length in motor neurons expressing $HSP60^{V72I}$ vs. $HSP60^{WT}$ vs. empty vector. Motor neurons were injected with plasmids encoding $HSP60^{WT}$, $HSP60^{V72I}$, or empty PCDNA3 vector, as well as a plasmid encoding pOCTeGFP to visualize the mitochondria. Live motor neurons were visualized and their axons imaged on day 3 or day 5 post-microinjection as described in materials and methods. Mitochondrial measurements were obtained using ImageJ and averaged for each axon. $HSP60^{V72I}$ expression did not cause obvious mitochondrial rounding, as mitochondria in all injection conditions appeared as a reticular network **(a)** nor did it cause a more subtle change in mitochondrial length at either time point **(b)** (day 3 P = 0.3235, day 5 P = 0.1980, One-way ANOVA). Day 3 data includes measurements from 268-365 mitochondria per condition (11-14 axons). Day 5 data includes measurements from 1206-2162 mitochondria per condition (51-73 axons).

Figure 5

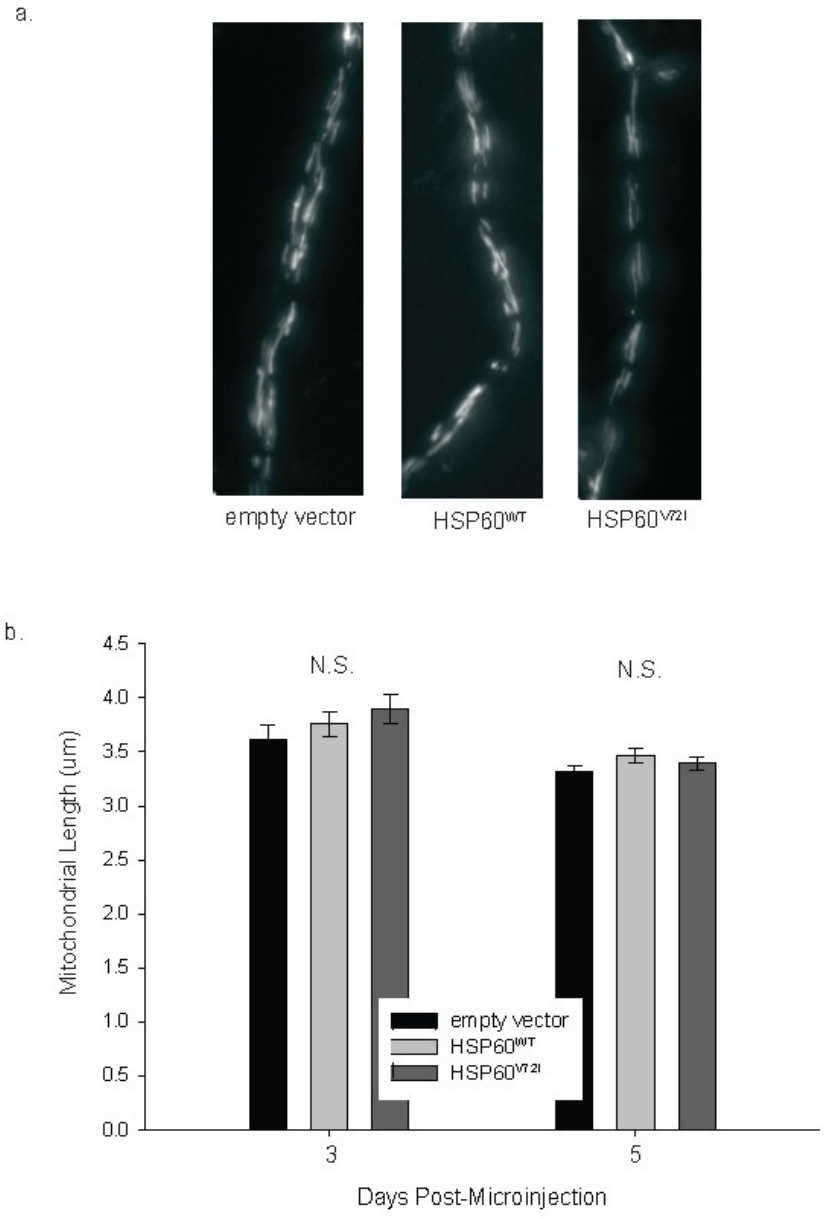

a.
empty vector
HSP60WT
HSP60V72I
b.
Mitochondrial Length (um)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
N.S.
N.S.
empty vector
HSP60WT
HSP60V72I
3
5
Days Post-Microinjection

Figure 6. Mitochondrial membrane potential ($\Delta\Psi$) in cultured motor neurons expressing human $Hsp60^{V72I}$ vs. $Hsp60^{WT}$ vs. empty vector. Motor neurons were injected with plasmids encoding $HSP60^{WT}$, $HSP60^{V72I}$, or empty PCDNA3 vector and a plasmid encoding GFP as a marker. On day 3 or 5 post-microinjection live motor neurons were loaded with the potentiometric dye TMRM, whose uptake is proportional to $\Delta\Psi$, and their processes were imaged **(a)**, as described in materials and methods. As a control, the uncoupling agent FCCP was added to some TMRM-treated cultures to show that a loss of $\Delta\Psi$ would, in fact, lead to a loss of TMRM from the mitochondria. FCCP-induced loss of $\Delta\Psi$ is shown in **(b)**, with GFP marker shown to demonstrate that images are in focus. Pixel intensity of the TMRM signal of individual mitochondria was obtained using MetaFluor and averaged for each motor neuron process. The TMRM signal was not significantly lower in motor neurons expressing $HSP60^{V72I}$ compared to those expressing $HSP60^{WT}$ or empty vector (Day 3 $P = 0.7340$. Day 5 $P = 0.3369$. One-way ANOVA) **(c).** Day 3 data includes measurements from 43-47 motor neurons per condition. Day 5 data includes measurements from 34-47 motor neurons per condition.

Figure 6

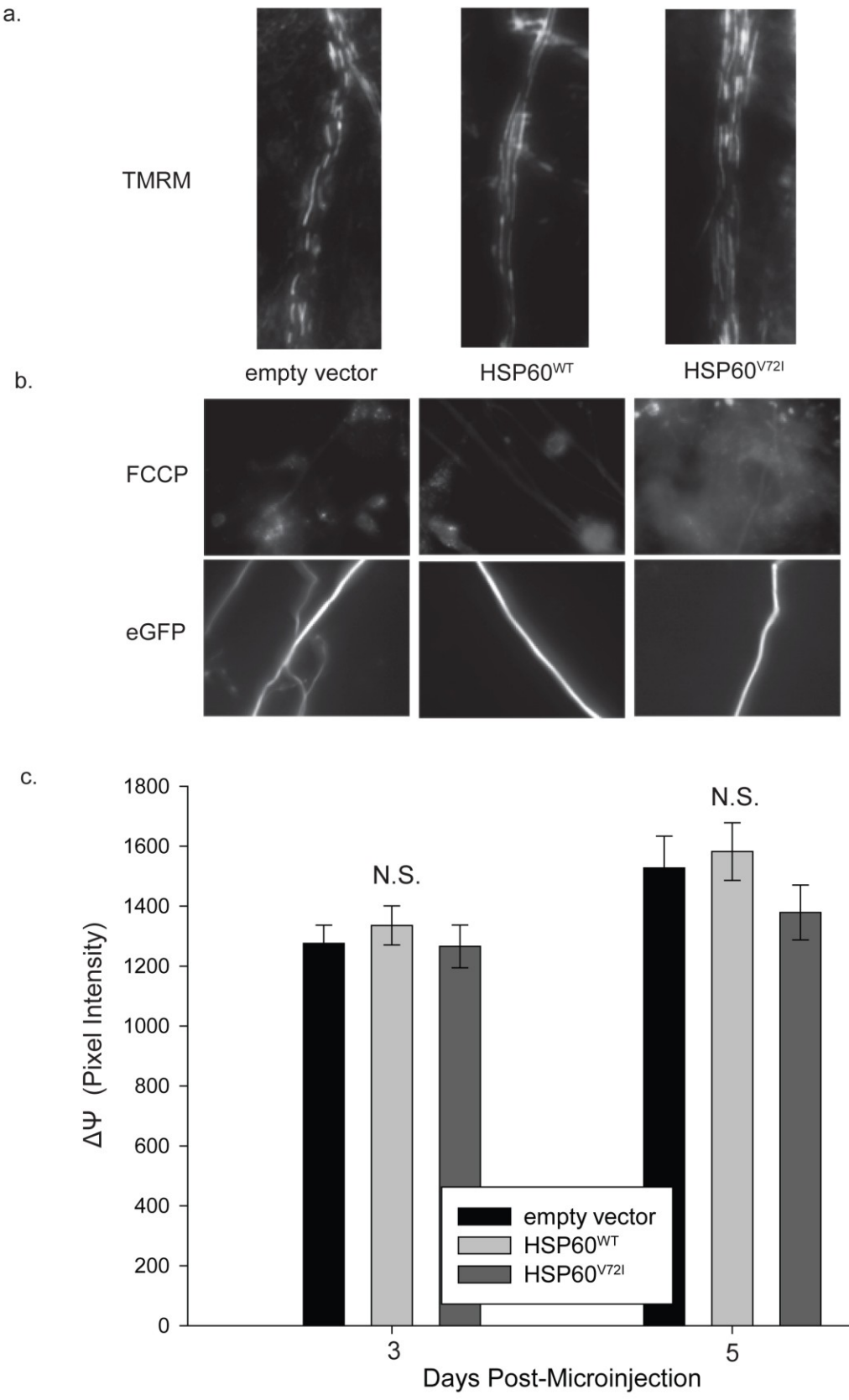

a.
TMRM
empty vector
HSP60WT
HSP60V72I
b.
FCCP
eGFP
c.
ΔΨ (Pixel Intensity)
1800
1600
1400
1200
1000
800
600
400
200
0
N.S.
N.S.
empty vector
HSP60WT
HSP60V72I
3
5
Days Post-Microinjection

Figure 7. Oxidative stress resistance of motor neurons expressing human $Hsp60^{V72I}$ vs. $Hsp60^{WT}$ vs. empty vector. Motor neurons were injected with plasmids encoding $Hsp60^{WT}$, $Hsp60^{V72I}$, or empty PCDNA3 vector, and a plasmid encoding GFP as a marker and counted for 3 days. On day 3 motor neurons were subjected to treatment with the superoxide generator paraquat dichloride, and viable motor neurons were counted at 12-hour intervals for 48 hours of paraquat treatment, as described in materials and methods. While paraquat treatment caused a general decline in motor neuron viability, the viability of oxidatively-stressed motor neurons expressing $HSP60^{V72I}$ was not significantly reduced compared to those injected with $Hsp60^{WT}$ or empty vector (empty vector vs. $Hsp60^{WT}$ P=0.4476; empty vector vs. $HSP60^{V72I}$ P=0.7999; $HSP60^{WT}$ vs. $HSP60^{V72I}$ P=0.3165, Wilcoxon log-rank test). Two to three cultures were injected per condition in two separate culture batches (for a total of 17 cultures), with 40-57 viable motor neurons per culture present on day 1.

Figure 7

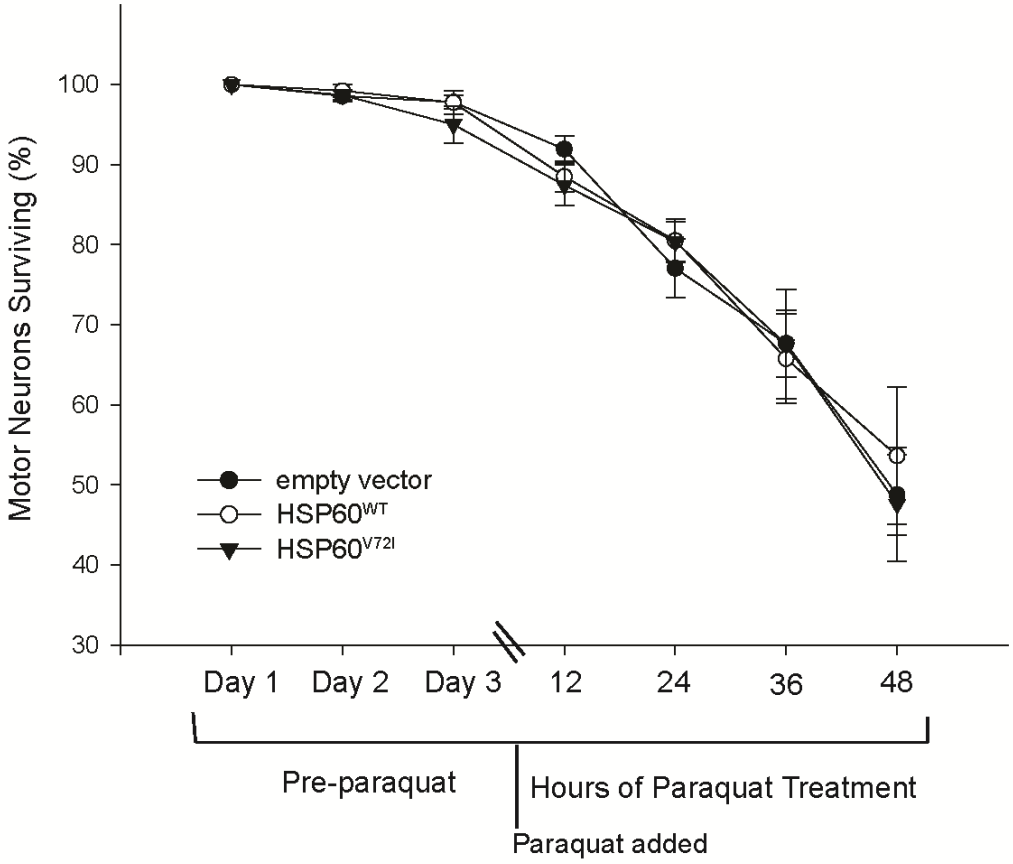

Motor Neurons Surviving (%)
100
90
80
70
60
50
40
30
empty vector
HSP60WT
HSP60V72I
Day 1
Day 2
Day 3
12
24
36
48
Pre-paraquat
Hours of Paraquat Treatment
Paraquat added

Figure 8. Axonal transport of mitochondria in motor neurons expressing human $Hsp60^{V72I}$ vs. $Hsp60^{WT}$ vs. empty vector. Motor neurons were injected with plasmids encoding $Hsp60^{WT}$, $Hsp60^{V72I}$, or empty PCDNA3 vector, as well as a plasmid encoding pOCTeGFP to visualize the mitochondria. Axons of live motor neurons were imaged as time-course series on day 3 or day 5 post-microinjection as described in materials and methods. Time-course images were used to create movies of mitochondrial movement using MetaFluor, and converted into kymographs using ImageJ **(a).** Kymographs were analysed to quantify the percentage of moving vs. stationary mitochondria in each axon. $Hsp60^{V72I}$ expression did not reduce the percentage of moving mitochondria in motor neuron axons compared to empty vector at either time point (Day 3 $P = 0.6355$. Day 5 $P = 0.3538$. One-way ANOVA) **(b)**. Day 3 data includes 9-13 axons per condition. Day 5 data includes 13-27 axons per condition.

Figure 8

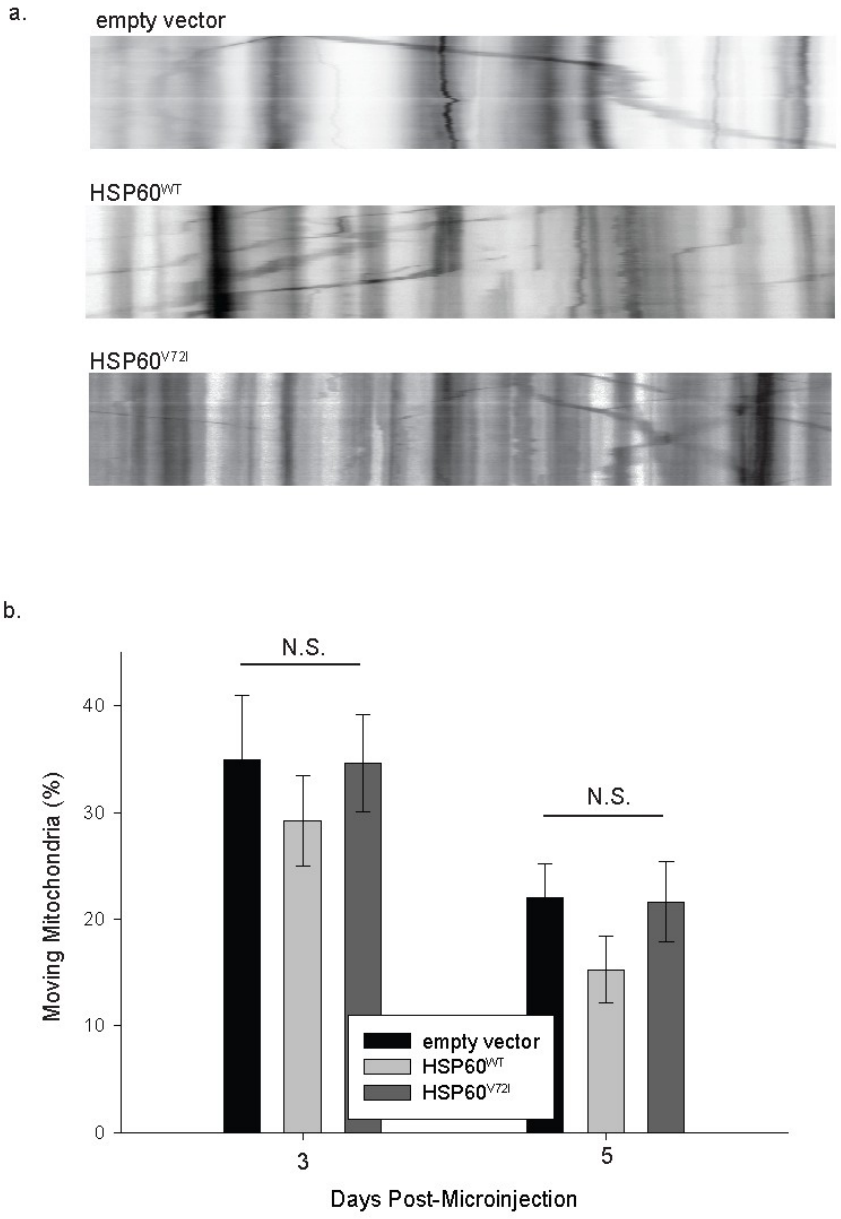

a.
empty vector
HSP60WT
HSP60V72I
b.
N.S.
N.S.
Moving Mitochondria (%)
0
10
20
30
40
3
5
Days Post-Microinjection
empty vector
HSP60WT
HSP60V72I

Figure 9. Expression of an ATPase deficient mutant Hsp60 ($HSP60^{D423A}$) does not reduce the viability of cultured motor neurons compared to a mutant Hsp60 implicated in hereditary spastic paraplegia SPG13 ($Hsp60^{V72I}$). Motor neurons were injected with plasmids encoding $Hsp60^{V72I}$ or $Hsp60^{D423A}$, using dextran-fluorescein as a marker and counted for 10 days. It was previously shown that the viability of motor neurons expressing $Hsp60^{V72I}$ was not significantly reduced compared to those expressing $HSP60^{WT}$. The ATPase deficient $Hsp60^{D423A}$ similarly caused no reduction in motor neuron viability compared to $Hsp60^{V72I}$. 2-3 cultures were injected per condition, with 21-38 motor neurons counted per culture on day 1.

Figure 9

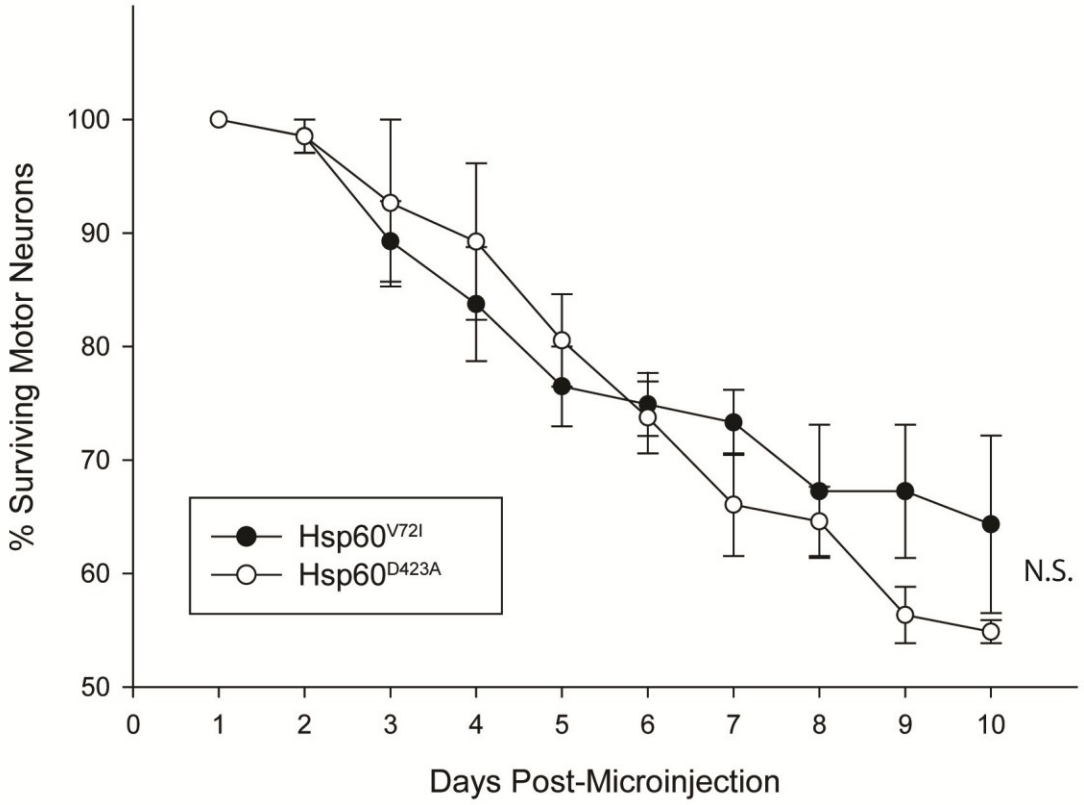

% Surviving Motor Neurons
100
90
80
70
60
50
0
1
2
3
4
5
6
7
8
9
10
Days Post-Microinjection
Hsp60V72I
Hsp60D423A
N.S.

Figure 10. Expression of $Hsp60^{D423A}$, an ATPase deficient mutant mitochondrial heat shock protein, does not cause mitochondrial shortening in cultured motor neurons. Motor neurons were injected with plasmids encoding $Hsp60^{V72I}$ or $Hsp60^{D423}$, as well as a plasmid encoding pOCTeGFP to visualize the mitochondria. Live motor neurons were visualized and their axons imaged on day 5 post-microinjection as described in materials and methods. Mitochondrial measurements were obtained using ImageJ and averaged for each axon. It was previously shown that $Hsp60^{V72I}$ expression did not cause obvious mitochondrial shortening compared to $Hsp60^{WT}$. $Hsp60^{D423A}$ expression also failed to induce changes in mitochondrial length ($P = 0.849673$, two-tailed t-test for independent samples). Data includes measurements from 1210-1320 mitochondria per condition (52-56 axons).

Figure 10

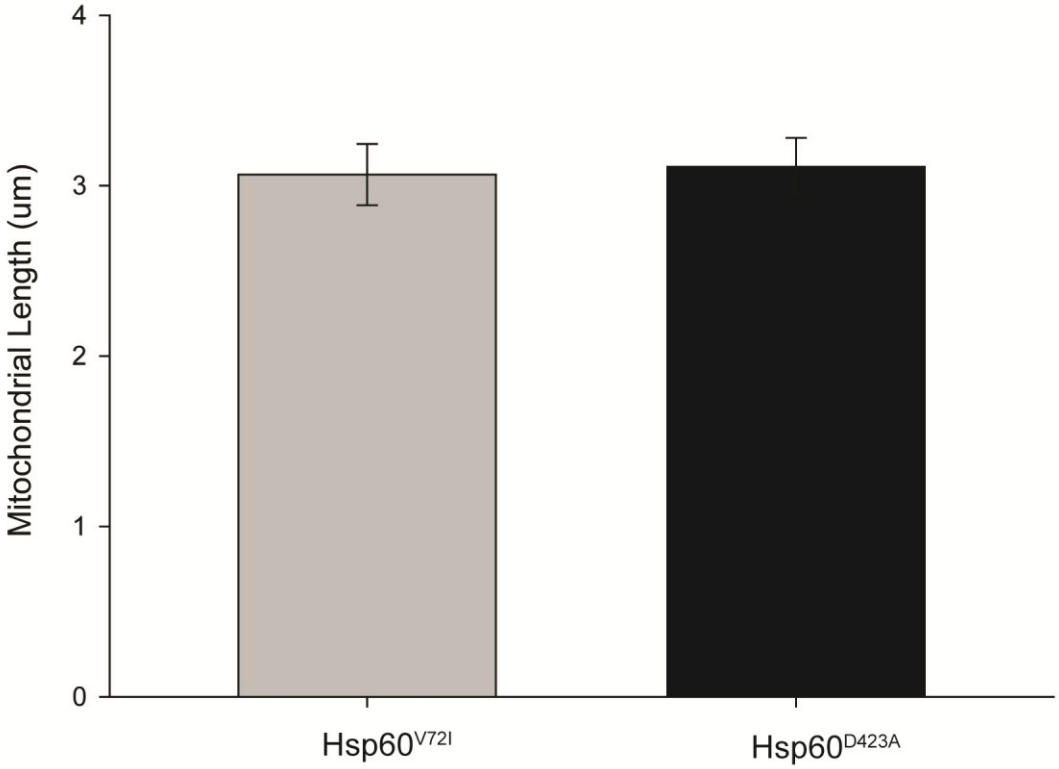
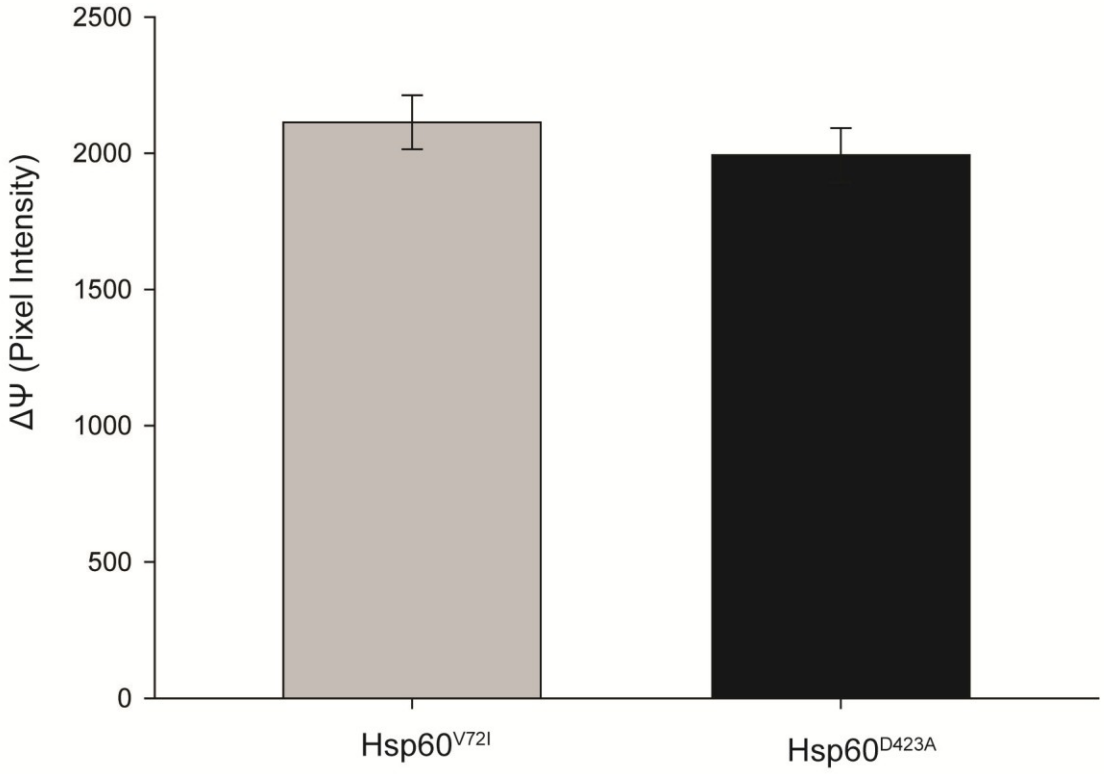


Figure 11. Expression of $HSP60^{D423A}$, an ATPase deficient mutant mitochondrial heat shock protein, does not reduce the mitochondrial membrane potential ($\Delta\Psi$) in cultured motor neurons. Motor neurons were injected with plasmids encoding $HSP60^{V72I}$ or $HSP60^{D423A}$, and a plasmid encoding GFP as a marker. On day 5 post-microinjection live motor neurons were loaded with the potentiometric dye TMRM, whose uptake is proportional to $\Delta\Psi$, and their processes were imaged as described in materials and methods. Pixel intensity of the TMRM signal of individual mitochondria was obtained using MetaFluor and averaged for each motor neuron process. It was previously shown that expression of $HSP60^{V72I}$ in motor neurons does not reduce the TMRM signal in mitochondria compared to $HSP60^{WT}$. Likewise, the TMRM signal was not significantly lower in motor neurons expressing $HSP60^{D423A}$ compared to those expressing $HSP60^{V72I}$ ($P = 0.398030$, two-tailed t-test for independent samples). Data includes measurements from 35-42 motor neurons per condition.

Figure 11


2500
2000
1500
1000
500
0
ΔΨ (Pixel Intensity)
Hsp60V72I
Hsp60D423A

CHAPTER 6 – Conclusions and Discussion

The work presented here investigated the mitochondrial heat shock protein, Hsp60, with two basic goals: To determine whether the wild-type form could be a potential therapeutic target for motor neuron survival in a primary culture model of fALS due to SOD1 mutation, and to determine whether a mutant form of Hsp60 said to cause a subtype of hereditary spastic paraplegia could be used to model potential mechanisms of mitochondrial dysfunction in this disease, using experimental techniques already well-established in modeling other neurodegenerative mechanisms in culture.

6.1 $Hsp60^{WT}$ in ALS

Given the early appearance of mitochondrial abnormalities in ALS pathogenesis, it is desirable to improve the defenses of mitochondria against insults such as the accumulation of misfolded proteins, for example mutant SOD1, oxidative stress, and calcium dysregulation, which could lead to mitochondrial dysfunction. One putative target for helping the mitochondria cope with the barrage of pathogenic insults inherent in the ALS cellular environment is to increase the expression of proteins involved in mitochondrial protein quality control, for example the mitochondrial heat shock proteins. Mitochondrial Hsps could protect mitochondria by interacting directly with mutant, misfolded SOD1 within mitochondria (although this represents only a small percentage of total aberrant SOD1 protein, or could protect through indirect mechanisms, for example the by maintaining proteins that make up the respiratory chain, which might be experiencing oxidative damage (Veereshwarayya et al., 2006).

In this study, the expression of endogenous human mitochondrial Hsp60 failed to prolong the viability of cultured motor neurons expressing the ALS-causing $SOD1^{G93A}$, and did not reduce more subtle signs of neurotoxicity, specifically, the mitochondrial

rounding and SOD1 inclusion formation characteristic of mutant SOD1 toxicity. It is unclear, however, whether or not endogenous human Hsp60 affects other, more subtle, indicators of mitochondrial distress, for example, mitochondrial membrane potential, ATP production, generation of reactive oxygen species, and activation of mitochondrial apoptotic pathways. Because increased Hsp60 expression did not improve motor neuron viability or general appearance of mitochondria, these detailed mechanisms were not examined.

Because the cultures used for these experiments express high levels of endogenous mouse Hsp60, the introduction of exogenous human Hsp60 may simply have had little impact because the exogenous protein "dosage" was not dramatically higher than the endogenous mitochondrial Hsp60 already available. It is also possible, given the reduction in ATP production caused by mutant SOD1 expression, for example by blockage of VDAC1 conductance, (Israelson et al., 2010) that the expressed Hsp60 was unable to work effectively to bind, re-fold and release misfolded proteins in the matrix, a process that is ATP-dependent and often requires cofactors, such as the small Hsp10 that acts as a "cap" on the Hsp60 double-ring structure, helping to change its conformation and regulating the ATPase cycle that allows for efficient binding and release of some, thought not all, proteins from the Hsp60 complex (Bukau and Horwich, 1998).

There are conflicting results in the literature on heat shock protein up-regulation and neuronal protection in models of ALS. For example, Kalmar and Greensmith (2009) observed that two pharmacological inducers of hsp expression, arimoclomol and celastrol, had opposing effects when used to treat cultured motor neurons exposed to pro-apoptotic and oxidative stressors, namely staurosporin or H_2O_2: While both drugs increased the levels of Hsp70 in culture, arimoclomol promoted motor neuron survival, while celastrol increased apoptotic motor neuron death. On the other hand, Kiaei et al. (2005) found that celastrol was neuroprotective and increased lifespan of $SOD1^{G93A}$ mice

in a dose-dependent manner. It has been suggested that perhaps celastrol provides neuroprotection *in vivo* by increasing hsp expression in cells other than the motor neurons, for example astroglia and microglia, or that its protective effects are due to its anti-inflammatory and/or antioxidant properties (Kalmar and Greensmith, 2009).

Given the conflicting results of Hsp up-regulation reported above, it is evident that increasing Hsps is not a panacea for neuroprotection in ALS. This point is emphasized by recent findings by Tradewell, et al. (2011) from a study investigating the relationship between mutant SOD1 protein aggregation, mitochondrial abnormalities, and calcium dysregulation in a primary dissociated spinal cord culture model of fALS. The expression of $SOD1^{G93A}$ in motor neurons led to increased mitochondrial calcium, decreased $\Delta\Psi$, and mitochondrial rounding, followed by increased endoplasmic reticular calcium, increased cytosolic calcium, and finally, the appearance of aggregated mutant SOD1. When cultures containing $SOD1^{G93A}$-expressing motor neurons were treated with the Hsp inducer, geldanamycin, protein aggregation and mitochondrial rounding were prevented; however, Hsp induction by geldanamycin did not prevent the decrease in $\Delta\Psi$, nor did it ameliorate calcium dyregulation. This sheds light on the possibility that not all mechanisms of mitochondrial dysfunction are directly related to problems with protein quality control, and therefore, Hsp-boosting therapies are likely to require co-therapies, for example, to buffer excess calcium, in order to truly preserve mitochondrial function and reduce subsequent neuronal death. How mitochondrial Hsps, such as Hsp60, might fit in with this picture is currently not clear.

Unfortunately, the expression of mitochondrial heat shock proteins, such as mtHsp70 and Hsp60, has not been measured in studies examining the pharmacological up-regulation of hsps in ALS models, which have typically focused on regulation of the heat shock transcription factor, Hsf1, and levels of cytosolic hsps like Hsp70, Hsp90, and Hsp27 (Kieran et al., 2004, Kiaei et al., 2005, Kalmar et al., 2008, Kalmar and

Greensmith, 2009). It is therefore unclear whether or not Hsp60 is contributing to neuroprotection, or lack thereof, in these models.

The current study examined increased Hsp60 expression in motor neurons, to the exclusion of other cell types in culture. Given the contribution of glial cells to disease pathogenesis in ALS, and the cytoprotection conferred by the glial heat shock response (Batulan et al., 2003), it would be important for future studies of Hsp60 in ALS to consider expression in non-motor neuron cells. It has already been shown that Hsp60 is upregulated in non-neuronal cells, especially in reactive astrocytes and macrophages, in tissues from patients with ALS (Martin et al., 1993), but whether or not this mechanism is neuroprotective remains to be clarified.

Finally, it should be noted that any potential therapeutic benefits of upregulating mitochondrial Hsps must be weighed against findings linking these protein chaperones with cancer. Aging *Drosophila* that over-expressed the small mitochondrial heat shock protein Hsp22 had longer lifespans, but the expression of *Drosophila* Hsp22 in human fibroblasts both increased the proliferation and lifespan of these cells, and increased their susceptibility to tumor formation (Morrow et al., 2010). Increasing Hsp60, similarly, may pose a risk for cancer, especially given its known pro-and anti-apoptotic functions, and its association with proteins that regulate the cell cycle (Bukau and Horwich, 1998, Samali et al., 1999, Xanthoudakis et al., 1999, Kirchhoff et al., 2002, Wadhwa et al., 2005). Hsp60 is indeed already being investigated as a bio-marker for the diagnosis, and target for treatment, of a variety of cancers, as levels of Hsp60 seem to increase with cancer severity (reviewed in Czarnecka et al., 2006, Cappello et al., 2008).

Though it was hoped that increasing mitochondrial Hsp60 would impart similar protective benefits to motor neurons as the expression of Hsp22 had in aging *Drosophila* (Morrow et al., 2004), this was not the case in our primary culture model of fALS1. It is

unclear at this time whether endogenous Hsp60 influences disease onset or progression. Future studies of heat shock proteins in ALS treatment should examine the mitochondrial Hsps along with the cytosolic Hsps to help clarify their potential role in neuroprotection.

6.2 Hsp60^{V72I} in Hereditary Spastic Paraplegia

Mutations in the mitochondrial heat shock protein, Hsp60, are said to cause a subset of hereditary spastic paraplegia cases. However, the mechanisms by which Hsp60 dysfunction leads to axonal degeneration and neuronal death remain unclear. The purpose of this study was to model hereditary spastic paraplegia SPG13 by expressing plasmids encoding the putative disease-causing mutant Hsp60^{V72I}in motor neurons of primary dissociated spinal cord culture, however, a clear phenotype was not obtained. The introduction of human Hsp60^{V72I} did not reduce the viability of cultured motor neurons, nor did it cause mitochondrial rounding or shortening, decrease mitochondrial membrane potential, increased vulnerability to oxidative stress, or compromise axonal transport of mitochondria. However, the expression of a severely ATPase-deficient mutant, human Hsp60^{D423A}, also failed to cause a phenotype, showing no effect on motor neuron viability, mitochondrial length, or $\Delta\Psi$. The most likely explanation for this lack of disease phenotype is that the cultures used for experiments express a high amount of endogenous wild-type mouse Hsp60, as indicated by strong labeling by anti-Hsp60 antibodies (mouse anti-Hsp60 StressGen SPA-806 or rabbit anti-Hsp60 SantaCruz SC13966, not shown), and the level of expression of Hsp60 mutants was not sufficient to exert a dominant-negative effect on the function of the endogenous protein. It is unknown whether mutant and wild-type Hsp60 protein subunits assemble equally well

together, as compared to the association of wild-type subunits with each other or mutant subunits with each other. Furthermore, it has been shown that Hsp60 chaperonins with only one functional ring are still able to accomplish mitochondrial protein folding *in vivo* (Nielsen and Cowan, 1998). Perhaps a higher concentration of injected Hsp60 mutant plasmid is needed to encourage a higher rate of mixed-ring and mutant-mutant ring assembly. The rate of Hsp60 turnover in these cultures is also unknown. It is possible that the existing pool of endogenous wild-type Hsp60 lasts throughout the course of the experiments and is relatively undiluted by the newly-synthesized exogenous Hsp60 mutant protein. Therefore, future experiments using primary culture to model potential mechanisms of neurodegeneration in hereditary spastic paraplegia SPG13 should include knock-down of the endogenous $Hsp60^{WT}$ pool. Creating cultures from Hsp60 knockout mice is not possible, as inactivation of the *HSPD1* gene is embryonic lethal (Christensen et al., 2010). However, a different strategy could involve the simultaneous microinjection of shRNA specific for mouse Hsp60, and plasmid encoding human $Hsp60^{WT}$, $Hsp60^{V72I}$, or $Hsp60^{D423A}$ with an inducible promoter, in order to stimulate exogenous Hsp60 expression after knock-down of the endogenous protein has been achieved.

Aside from itself being a rare cause of hereditary spastic paraplegia, Hsp60 mutations have also been implicated as a genetic modifier of the disease, potentially interacting with, and worsening the effects of spastin mutation, which is a prominent cause of the disease (Depienne et al., 2007, Hewamadduma et al., 2008). It was found that while patients with a known spastin deletion exhibited a pure form of autosomal-dominant hereditary spastic paraplegia (SPG4), the presence of a spastin deletion in

combination with the Hsp60^{G563A} mutation resulted in a significantly earlier age of onset (5.7 years +/- 1.5 SEM vs. 26.5 years +/- 4.6 SEM for patients without Hsp60 mutation). Though Hsp60^{G563A} polymorphism was present in 5 out of 262 control chromosomes, its presence alone did not cause disease. The mechanism by which Hsp60 mutation might speed the onset of SPG4 remains unknown.

Another mechanism of motor neuron dysfunction that was not explored in the present work is the potential involvement of Hsp60^{V72I} in disrupting myelination. Another Hsp60 mutant, The Hsp60 ^{D29G} mutation, causes mitCHAP-60 disease, an autosomal recessive disorder involving brain hypomyelination and leukodystrophy (Magen et al., 2008). In the experimental autoimmune encephalomyelitis animal model of multiple sclerosis, a disease in which inflammation and demyelination prominently feature, Hsp60 was highly expressed at lesion sites, especially in infiltrating oligodendrocytes and astrocytes. Interestingly, Hsp60 at chronic lesion sites was cytoplasmically-localized, whereas Hsp60 at non-lesion sites remained mitochondrial (Gao et al., 1995). It is possible that mutations in Hsp60 might change its pattern of localization in the cell, which might in turn affect its protein-folding functions or immunomodulating capabilities, especially at sites of neuroinflammation. Unfortunately, the primary culture model being used does not lend itself to studies of myelination, as little myelination occurs in dissociated spinal cord cultures.

6.3 Closing Remarks

By examining potential neuroprotective effects of Hsp60 in a culture model of SOD1-associated fALS, especially at the level of mitochondria, (hypothesis 1), and, on the other hand, attempting to model HSPG13-related mitochondrial dysfunction in culture through the expression of a HSPG13-associated Hsp60 mutant, (hypothesis 2), it was hoped that this work would more clearly define the function of Hsp60 in motor neurons more generally, and to help elucidate potential common pathways of mitochondrial dysfunction that contribute to multiple neurodegenerative diseases. However, much work remains to be done to achieve these objectives, especially with regards to creating a culture model of HSPG13 in which a phenotype, if present, can actually be observed, and validating the association of mutations in Hsp60 in this disorder.

CHAPTER 7 –References

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