8-2013

STUDYING AGGREGATE FORMATION BY AMYOTROPHIC LATERAL SCLEROSIS-ASSOCIATED MUTANT SOD1 PROTEIN IN DROSOPHILA MODEL

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STUDYING AGGREGATE FORMATION BY AMYOTROPHIC LATERAL SCLEROSIS-ASSOCIATED MUTANT SOD1 PROTEIN IN *DROSOPHILA* MODEL

A

THESIS

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

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August 2013

ABSTRACT

A common pathological hallmark of most neurodegenerative disorders is the presence of protein aggregates in the brain. Understanding the regulation of aggregate formation is thus important for elucidating disease pathogenic mechanisms and finding effective preventive avenues and cures. Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is a selective neurodegenerative disorder predominantly affecting motor neurons. The majority of ALS cases are sporadic, however, mutations in superoxide dismutase 1 (SOD1) are responsible for about 20% of familial ALS (fALS). Mutated SOD1 proteins are prone to misfold and form protein aggregates, thus representing a good candidate for studying aggregate formation.

The long-term goal of this project is to identify regulators of aggregate formation by mutant SOD1 and other ALS-associated disease proteins. The specific aim of this thesis project is to assess the possibility of using the well-established *Drosophila* model system to study aggregation by human SOD1 (hSOD1) mutants. To this end, using wild type and the three mutant hSOD1 (A4V, G85R and G93A) most commonly found among fALS, I have generated 16 different SOD1 constructs containing either eGFP or mCherry in-frame fluorescent reporters, established and tested both cell- and animal-based *Drosophila* hSOD1 models. The experimental strategy allows for clear visualization of ectopic hSOD1 expression as well as versatile co-expression schemes to fully investigate protein aggregation specifically by mutant hSOD1.

I have performed pilot cell-transfection experiments and verified induced expression of hSOD1 proteins. Using several tissue- or cell type-specific Gal4 lines, I have confirmed

the proper expression of hSOD1 from established transgenic fly lines. Interestingly, in both *Drosophila* S2 cells and different fly tissues including the eye and motor neurons, robust aggregate formation by either wild type or mutant hSOD1 proteins was not observed. These preliminary observations suggest that *Drosophila* might not be a good experimental organism to study aggregation and toxicity of mutant hSOD1 protein. Nevertheless this preliminary conclusion implies the potential existence of a potent protective mechanism against mutant hSOD1 aggregation and toxicity in *Drosophila*.

Thus, results from my SOD1-ALS project in *Drosophila* will help future studies on how to best employ this classic model organism to study ALS and other human brain degenerative diseases.

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CHAPTER ONE

Introduction

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that predominantly and selectively affects upper and lower motor neurons in the brain and spinal cord. ALS, in the US is more commonly known as Lou Gerig's disease and is diagnosed at a rate of about 5,600 new cases a year, affecting a total of 30,000 Americans at any given time ("ALS association," 2013). The pathology of this disorder is characterized by a deterioration of the neuromuscular junction (NMJ), caused by the retraction and progressive degeneration of motor neuron axons (healthy NMJ is seen in figure 1). This results in sensory loss, muscle weakness, muscle atrophy and eventually paralysis. In human patients ALS symptoms onset is varied but usually begins with sporadic weakness or stiffness and then progresses to systemic weakness and loss of function. Paralysis and a complete loss of mobility occur at advanced stages of disease and eventually dysfunction of respiratory motor neurons occurs. Death is usually the result of paralyzed respiratory muscles and occurs within 1 to 5 years of diagnosis, but has been document to take up to 20 years after diagnosis (Bruijn, Miller, & Cleveland, 2004). Although, in later stages total loss of motor function is very common, control of eye muscles and movement as well as bladder function often remains unaffected.

Figure 1. Healthy motor neuron and neuromuscular junction.

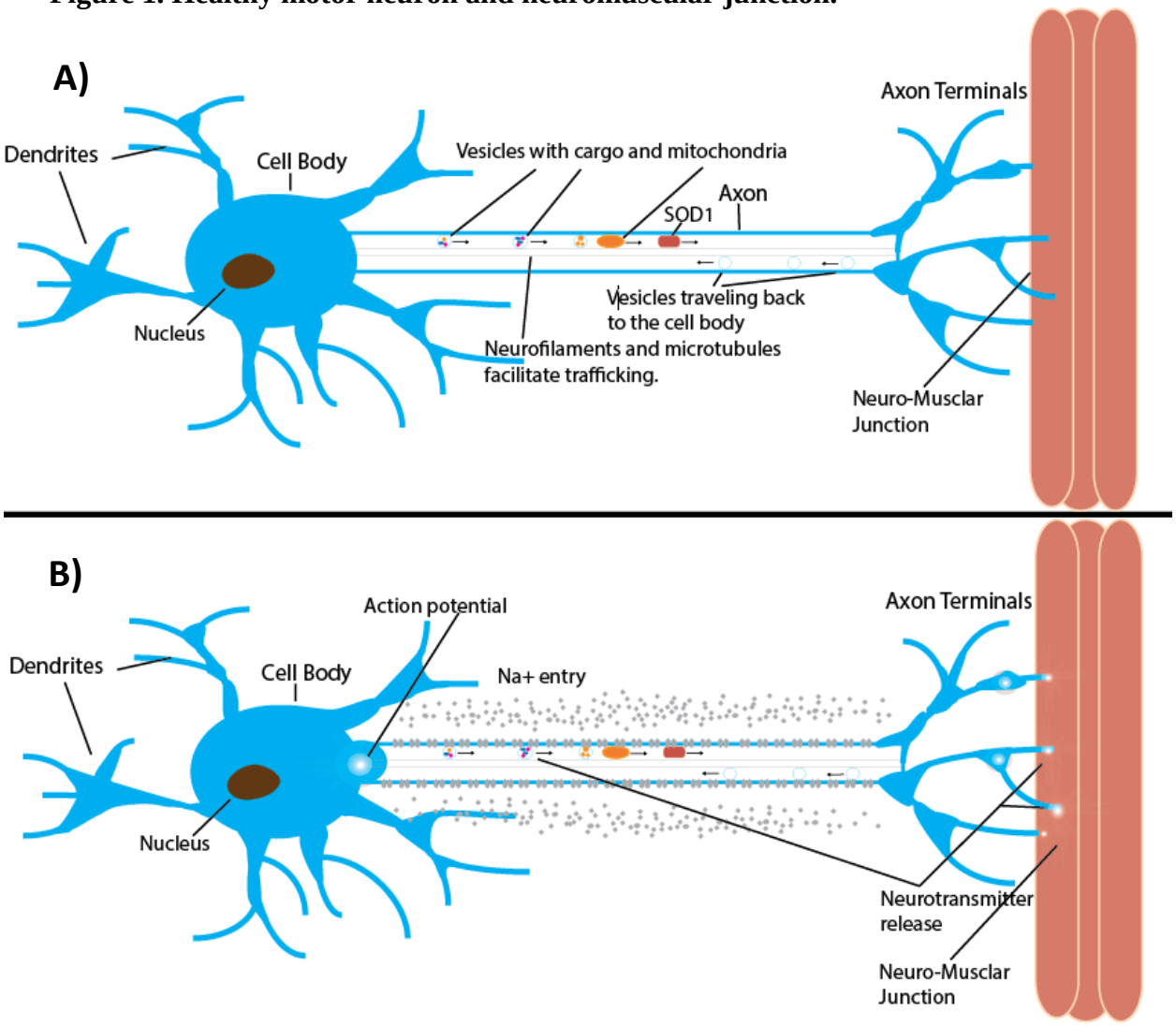


Figure 1. Healthy motor neuron and neuromuscular junction.

This is a diagram of a typical motor neuron and its connection to the neuromuscular junction (NMJ).

Image (A) shows a basic understanding of vesicle, protein and mitochondria trafficking to and from axon terminals.

Image (B) depicts action potential generated at the soma and propagated down the neuron with sodium entry towards the NMJ. That action potential provides the necessary cytosolic increase in calcium required to facilitate neurotransmitter release

Genetic basis of ALS

Most cases of ALS are sporadic with no known genetic component, 5 to 10% of ALS is familial. Among the familial Amyotrophic Lateral Sclerosis (fALS) cases, around 20 – 40% are caused by one of over 150 identified mutations in the *superoxide dismutase 1* (SOD1) gene and about 50% of fALS are due to a hexanucleotide repeat expansion mutation in an intron of the *C9orf72* gene. Interestingly around 44 mutations in the *TDP-43* gene, which codes for an RNA binding protein, are responsible for about 5% of all sporadic and familial ALS (Da Cruz & Cleveland, 2011; Kwiatkowski et al., 2009; Mackenzie, Rademakers, & Neumann, 2010). However, a total of 46 identified mutations so far in the *Fused in Sarcoma* or *Translocated in liposarcoma* (FUS/TLS) gene, another RNA binding protein, is seen in around 4% of familial ALS, but rarely seen in sporadic ALS (Da Cruz & Cleveland, 2011; Vance et al., 2009).

Superoxide dismutase (SOD)

Among the many ALS-associated genes, SOD1 is the first identified and by far most extensively studied. In mammalians, there are three different superoxide dismutase enzymes and they are found at three different cellular locations. ALS-associated superoxide dismutase 1 (SOD1) is a cytosolic enzyme that causes the dismutation of the highly reactive O_2^- anion known as superoxide, to H_2O_2, which then can be further reduced to water (H_2O) and molecular oxygen (O_2). Superoxide dismutase 2 (SOD2) resides in the mitochondria while superoxide dismutase 3 (SOD3) is an extracellular protein. Notably, mutations in SOD2

have also been found to cause motor neuron disease and to exacerbate ALS caused by SOD1 mutations (van Zundert, Izaurieta, Fritz, & Alvarez, 2012).

ALS-associated mutation in SOD1

SOD1 itself encodes a small protein of 153 amino acids (a.a.) long and is fully functional as a homodimer. Remarkably, over 150 mutations distributed throughout this small protein have been identified in ALS patients. Among the most well characterized mutations in SOD1, an alanine (A) to valine (V) change at position 4 (A4V) is the most common mutation in fALS patients, and a glycine (G) to alanine (A) change at position 93 (G93A) was employed to create the first established mouse ALS model (van Zundert et al., 2012). Notably, SOD1 proteins harboring either of these two mutations are still enzymatically active as superoxide dismutase, although they become physically unstable and prone to aggregation. Another commonly modeled SOD1 mutation is a glycine (G) to arginine (R) change at position 85, referred to as G85R, which is commonly referred to as enzymatically inactive, although detailed studies suggest it retains a very low level of activity. However, SOD1 protein with this G85R mutation is more unstable as compared to other SOD1 mutants and aggressively forms aggregates (Bruijn et al., 1997; Wang et al., 2009).

Thus, far only one mutation in SOD1, D90A, has been seen to cause fALS in a recessive manner, while all other mutations cause fALS in a dominant manner. The recessive mutation of SOD1 is predominantly seen in a small subset of the Scandinavian population and appears to be recessive only in that population (Robberecht et al., 1996).

SOD1-based ALS models in mice

Modeling of SOD1 mutations related to ALS in mice has been successful in that it produces animals with consistent phenotypes that accurately reflect the disease course in humans. One exception is that in ALS mouse models, the onset of weakness and paralysis presents very consistently in the hind limbs, in contrast to the sporadic or non-uniform onset of the symptoms in human patients. Also, among the reported ALS mouse models, different disease relevant mutations of SOD1 cause consistent and nearly uniform symptoms. With variation in disease course mostly limited to the age of onset and length of disease course, making findings in one model relevant to other models. Two exceptions are notable, first G127X mutation in SOD1 induces symptoms at low expression levels, although still accumulates in CNS tissue and results in both protoaggregates and aggregates. It is in contrast with other more common ALS models that mostly rely on overexpression of mutated SOD1 (Jonsson et al., 2004). Second, A4V mutation in SOD1 appears to require either a high level of protein expression, a common feature of mutant SOD1models, or co-expression with wild type SOD1(Deng et al., 2006).

Mutant SOD1 induces a gained of toxicity

The pathogenic mechanisms underlying ALS are still far from clear (see Chapter 2 below). The reason SOD1 mutations are so tightly linked to ALS is also not yet known. However, SOD1-associated ALS in general is believed to be the result of a gain of toxicity in mutant SOD1, because many mutant forms of the protein remain catalytically active. In addition, for those mutations that do not retain the endogenous SOD1 function, they do not correlate with correspondingly altered severity of the symptoms or an earlier onset of the disease (Boillee, Vande Velde, & Cleveland, 2006; Bruijn et al., 2004; Ince et al., 2011; Pasinelli & Brown, 2006). Moreover, mice with SOD1 knock out do not develop motor

neuron degeneration symptoms, but instead have been found to have increased incidence of tumor formation, although this does not impact survival or accelerate aging biomarkers (Boillee, Vande Velde, et al., 2006; Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007; Reaume et al., 1996).

CHAPTER TWO

Pathogenic Mechanisms of ALS: A Literature Review

Protein aggregates: a common pathological feature of many neurodegenerative diseases

Amyotrophic Lateral Sclerosis, Parkinson's, Alzheimer's, transmissible spongiform encephalopathy's and Huntington's are neurodegenerative diseases that affect different areas of the brain and involve different gene mutations. Nevertheless, they all involve the gradual loss of neurons. At the pathological level, almost all brain disease share a common feature: the formation and presence of aberrant protein deposits (aggregates).

It is generally believed that protein aggregates are derived from accumulation of mis-folded proteins. The protein aggregates are composed primarily of unique proteins in different diseases. For example, in Parkinson 's disease (PD), the Lewy body is mainly composed of PD-associated α-Synuclein protein; In Huntington's disease (HD), the inclusions contain the cleaved and mis-folded disease gene product Huntingtin; In Alzheimer's disease (AD), the extracellular plaques are predominantly consisted of secreted Aβ peptides derived from processed AD gene product APP, while the intracellular tangles are from the hyper-phosphorylated Tau protein. Similarly, the protein products of the ALS-associated genes such as mutant SOD1, TDP43 or FUS have also been found in neuronal aggregates from ALS patients (Bruijn et al., 2004). The close link between protein aggregates and brain degenerative diseases has promoted speculations of a causative relationship between abnormal protein deposition and neurodegenration.

Protein aggregation is a simple term for a highly complex process that involves a dynamic equilibrium between soluble monomers or oligomers of accumulated protein and various states of higher order insoluble structures termed aggregates. The path from

accumulated protein to aggregate is dependent on cellular environment, interaction with other proteins and concentration of the accumulated protein (Aguzzi & O'Connor, 2010). In the case of SOD1 associated fALS, there is an aberrant accumulation of mutant SOD1 protein accompanied by a lack of degradation by the ubiquitin mediated degradation pathway, resulting in aggregated inclusions consisting of mis-folded mutant SOD1 protein, as depicted in **figure 4** below (Bruijn et al., 2004). It has also been suggested the protein half-life plays a role in SOD1 mutations ability to aggregate aggressively and might be an indicator of aggregate formation (Wang et al., 2003).

However, there is a growing body of empirical evidence to suggest the actual formation of protein fibrils may be either neuroprotective or the end stage by-product of the real culprit. That is, earlier stage prefibrillar oligomers or protofibrils have been implicated as the true source of toxic element responsible for neuronal cell death (Caughey & Lansbury, 2003). It has been suggested that accumulated mutant proteins could overload protease machinery, overtax heat shock proteins and damage mitochondria, increasing the risk of cell death due to oxidative stress and thus inducing apoptosis. As an example of the complexity of the field, one study that illustrates the toxicity of pre-aggregate oligomers of mutant SOD1 in ALS focused on the G127X mutation that will produce a truncated SOD1 protein. This study found that mutant G127X SOD1 will cause ALS, even with fewer aggregates and at lower expression levels than other SOD1 mutants, which suggests that an overtaxing or sequestering of chaperones does not occur in some cases (Jonsson et al., 2004). Further, it has been shown that mis-folded mutant G127X SOD1 can exist at higher steady state levels between synthesis, mis-folded accumulation and degradation/aggregation in the central

nervous system, than elsewhere in an organism, suggesting a possible reason for CNS susceptibility to disease (Jonsson et al., 2004).

The above findings regarding the G127X mutation of SOD1 underscore the possibility that oligomers and protofibrils may be more damaging than fully formed aggregates. Sorting out the true cause of neurodegeneration and understanding the cellular regulation process of forming aggregates will be critical to developing effective treatments for ALS.

Astrocytes and Glia Cells: A brief introduction

Although ALS is a motor neuron disease, increasing evidence suggest an essential role of neuronal supporting cells especially astrocytes and glia in the disease pathogenesis (Nagai et al., 2007; Perea & Araque, 2005; Rao & Weiss, 2004; Wallis, Zagami, Beart, & O'Shea, 2012). Glia cells fall into two basic classifications based on their location they are either central glia or peripheral glia. Central glia cell types include microglia, astrocytes, oligodendrocytes and ependymal cells. Schwann cells are the only type of peripheral glia cells; they sheath axons and cover the cell bodies of neurons (Hammond, 2008).

Astrocytes were first believed to only be "brain stuffing" or the glue and structure, which provided the physical support that facilitated the organization and connections of neurons and to supply nutrients to neurons via liaison with and maintenance of the blood brain barrier (Volterra & Meldolesi, 2005). In the late 1980s it was discovered that astrocytes expressed voltage gated ion channels as well as receptors for neurotransmitters, suggesting they played a more sophisticated role in neural tissue than previously thought (Volterra & Meldolesi, 2005).

Prevalent dogma in the neuroscience field also held that astrocytes and glia cells could not participate in neuronal communication because they do not generate an action potential when excited. Now it is known that astrocytes and glia are excitable without the ability to generate an action potential, which has given new depth to the complexity of communication occurring in the brain (Bezzi & Volterra, 2001). Even without generating an action potential astrocytes and glia are capable of excitation, which results in the release of neurotransmitters to neighboring cells (Zonta, Angulo, et al., 2003; Zonta, Sebelin, et al., 2003). Further astrocytes and glia are active communicators in neuronal circuits with exocytosis of synaptic-like microvesicles containing neurotransmitters, such as glutamate, has been documented and diagramed in **figure 2** (Matsui & Jahr, 2003, 2004). The excitation of both glia and astrocytes can be quantified as a transient increases and subsequent decrease in calcium concentration (Zonta, Sebelin, et al., 2003). The two predominant forms of excitation have been documented and are either the result of chemical signals released by local neuronal circuits or can occur spontaneously in the absence of a canonical signal or trigger (Volterra & Meldolesi, 2005). Importantly, when astrocytes or glia display excitation through an increase in cytosolic calcium concentration they can directly excite adjacent neurons (Fellin et al., 2004). This knowledge lead to the discovery that astrocytes play an important role in many processes, including the formation and function of synapses as well as adult neurogenesis and in maintaining the vascular tone of the brain (Bushong, Marton, & Ellisman, 2004; Bushong, Martone, Jones, & Ellisman, 2002; Derouiche & Frotscher, 2001).

Figure 2. Astrocytes play an active role in synaptic cleft traffic and neuron response.

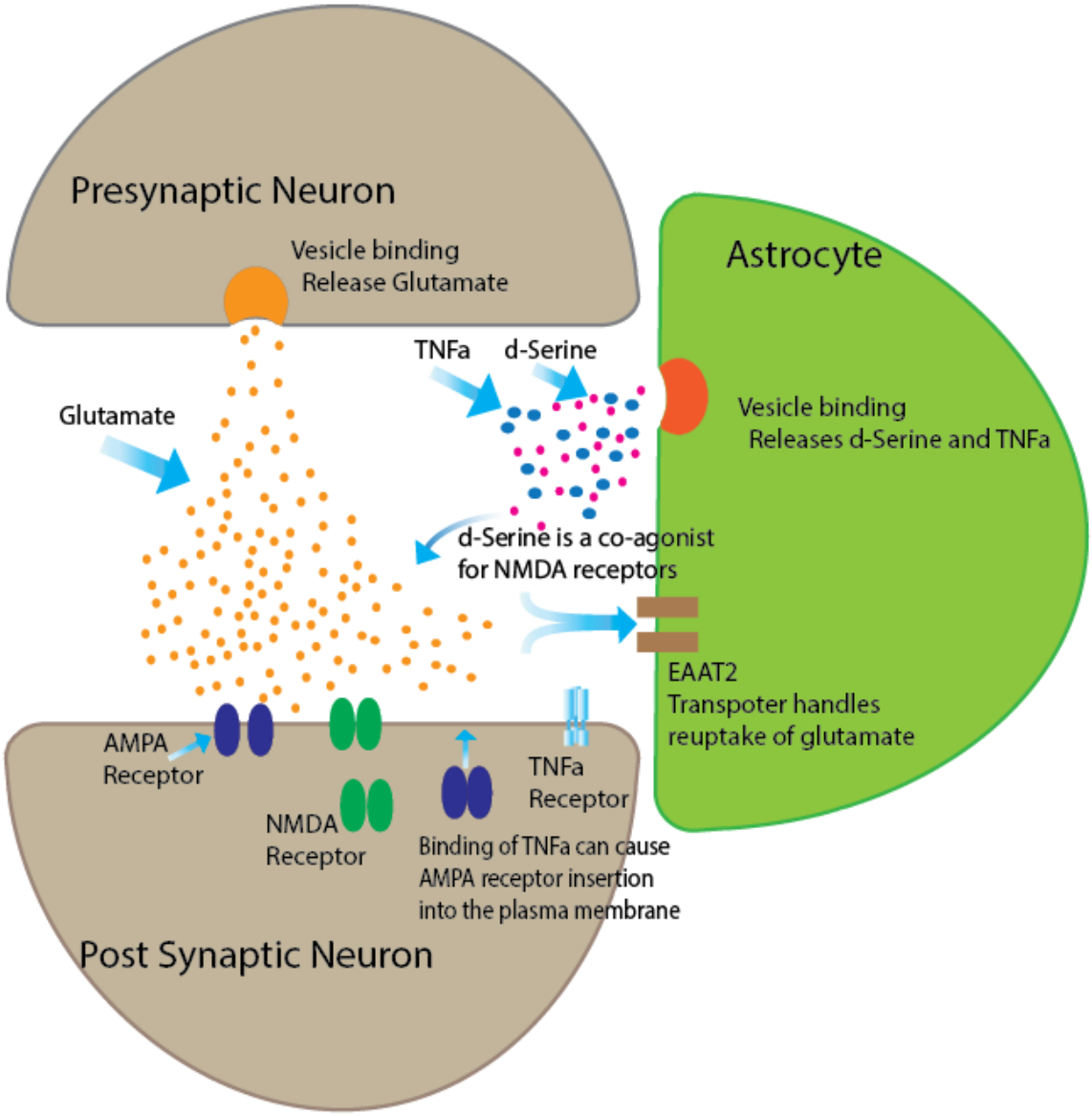


Figure 2 Astrocytes play an active role in synaptic cleft traffic and neuron response. Glutamate released from the pre-synaptic neuron travels across the cleft to activate the AMPA and NMDA receptors on the post synaptic cleft.

If glutamate is not cleared from the synaptic cleft by the EAAT2 glutamate reuptake transporter in the neighboring astrocytes, it will remain in the cleft and bind more receptors that are present or inserted into the membrane. Potentially inducing excessive signaling and excitoxicity.

Astrocytes have been shown to be involved in almost every functional aspect of the central nervous system from neurogenesis and synaptogenesis, to acting as local integration units and liaisons between synaptic and non-synaptic communication (Denise et al., 2004; Mauch et al., 2001; Sanai et al., 2004; Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001). The role of glia cells in neuronal signaling was also underestimated, now it is known that glia cells can regulate synaptic function and structure (Mauch et al., 2001; Ullian, Sapperstein, Christopherson, & Barres, 2001).

There are two documented examples where glia-cell signaling, to the neuron, directly regulates synaptic function and structure. The first includes Bergmann glial cells, where a calcium concentration increases resulting from glutamate activated AMPA receptors, maintains the extensive network of synapses that innervate Purkinje cells(Volterra & Meldolesi, 2005). The absence of this glia signal alters the hyperinnervation and synaptic current of the postsynaptic targets. (Iino et al., 2001) The second example is the binding of ephrin A3, which is released from the surface of astrocytic processes, to its receptor EphA4, which is expressed on dendritic spine membranes. This event initiates intracellular signals that control the shape, size and number of dendritic spines. Without this signal spines will grow too long and become very disorganized (Murai, Nguyen, Irie, Yamaguchi, & Pasquale, 2003). This information sets new precedents for understanding how information flow and trafficking in neuronal networks is established by excitation of both neurons and glia and astrocytes. It also serves to bring attention to the ability of astrocytes/glia to dynamically and extensively respond to the state of neurons and synaptic communication.

Astrocytes and Glia Cells: Role in ALS

Astrocytes inclusions, as they relate to ALS were described by Beckman *et al.* in a study that attempts to establish a timeline for motor neuron degeneration. The inclusion resembled the lewy bodies found in Parkinson's and were found to be the first abnormalities in G85R mutant hSOD1 expressing mice(Beckman, Estevez, & Crow, 2001). This occurred in the mutant mice at 8 to 10 months of age and was seen before inclusions were detected in motor neurons. Astrocyte localization was confirmed with immunoreactivity to antibodies specific to glial fibrillary acid protein (GFAP), which is not expressed in neurons. A two fold increase in SOD1 concentration was seen in the spinal cords of dissected mice along with a 10 fold increase in SOD1-G85R containing astrocyte inclusions at end stage and this was accompanied by a marked increase in astrocytosis.

Aggregates of SOD1 were then seen only in a few neurons before onset of symptoms, but were diffuse and small, though they were reactive to SOD1 and ubiquitin antibodies. At later stages of disease large round and irregular shaped SOD1 containing inclusions were observed that were immunoreactive to SOD1 antibodies at their core and periphery. These inclusions were reactive to ubiquitin, however only at the periphery. In the final stage of disease spinal cord extracted showed a 60% loss of motor axons and a 10 fold increase in mutant SOD1 astrocyte inclusions over motor neuron inclusions. Indicating that the astroglia are a major focus of damage for ALS associated SOD1 mutation G85R (Beckman et al., 2001).

Microglia cells are myeloid-lineage immune cells of the central nervous system. They and astrocytes are the primary inflammatory response mediators. Microglia has been

shown in some cases to cross the blood brain barrier (BBB) and or the blood spinal cord barrier to communicate and interact directly with the immune system (Boillee, Yamanaka, et al., 2006; Skaper & Facci, 2012; Weydt & Moller, 2005). Mast cells are bone marrow derived innate immune system cells that are capable of crossing the blood brain barrier and blood spinal cord barrier in the absence of inflammation. They are capable of producing many inflammatory and neurotrophic mediators, key to ALS are tumor necrosis factor-α (TNFα) and nerve growth factor, to which glia cells may respond (Skaper & Facci, 2012).

Neuron death can be caused by microglia activation and secretion of toxic factors such as TNFα and nitric oxide (NO) can be released from astrocytes expressing mutated SOD1 proteins, which encourages pro-apoptotic pathways in motor neurons (Volterra & Meldolesi, 2005). Release of toxic factors from astrocytes and glia ultimately kill motor neurons through the activation of BAX mediated cell death agents (Nagai et al., 2007). Importantly, it has been shown that among the many non-neuronal cells astrocytes are the only cell type that are capable of releasing such toxic factors and motor neurons are the only type of neuronal cells that are killed by these toxins (Nagai et al., 2007).

Motor neuron death also involves excitotoxicity which may be mediated by astrocytes, as they are the major mediators of neuronal support. Astrocytes are responsible for the regulation of ion concentrations in the extracellular space, as well as modulation and clearance of neurotransmitters, such as glutamate, from the synaptic cleft (Benediktsson, Schachtele, Green, & Dailey, 2005; Hirrlinger, Hulsmann, & Kirchhoff, 2004; Volterra & Meldolesi, 2005).

In ALS there are reports of the focal loss of the EAAT2 transporter (normal function seen in **figure 2**) in astrocytes, which is the dominant transporter responsible for glutamate reuptake. Glutamate is the major excitatory neurotransmitter (Parpura et al., 1994) and with the reuptake of glutamate inhibited, glutamate is left in the synaptic cleft free to bind to any available receptors. This will result in the activation of a cation channels that will depolarize the neuron and can ultimately result in repetitive neuronal excitability, calcium entry and eventually excitotoxicity through repetition. In addition to glutamate excitotoxicity as a result of the loss of EAAT2 transporter in astrocytes, the release of D-serine from glia, which causes chronic activation of NMDAR, has also been linked to mutant SOD1 and ALS (Sasabe et al., 2007).

Mitochondria dysfunction in ALS

Mitochondria are classically described as the power house of the cell and as such it is the primary source of ATP production. To achieve this vital role, it employs a very complex system that utilizes three partitioned domains which are the matrix, inner membrane space and the outer membrane. Using a set of intramembranous protein complexes in located in their inner membrane, mitochondria are able to establish and maintain and electron transport chain (ETC) for the purpose of stripping electrons and collecting protons (H^+ atoms) from molecules as seem in **figure 3**.

Notably, SOD1 is the major cytosolic protein present to prevent oxidative damage and reduce superoxide leakage form mitochondria. It has also been shown that SOD1 is present in the mitochondria, primarily in the inter membrane space but also in the matrix and on the cytosolic face of the outer membrane. (Carri & Cozzolino, 2011). The presence of

SOD1 is thought to provide protection to mitochondrial proteins from oxidation thus preserving mitochondrial homeostasis (Aquilano, Vigilanza, Rotilio, & Ciriolo, 2006; Kloppel, Michels, Zimmer, Herrmann, & Riemer, 2010; O'Brien, Dirmeier, Engle, & Poyton, 2004). Accordingly, mutations in SOD1 and its abnormal aggregation have been suspected to disrupt this important cellular protective mechanism and contribute to the disease pathogenesis.

Axonal transport of mitochondria has been shown to be dysfunctional in ALS and could indeed be related to the problems caused by mutant SOD1 presence in mitochondria (Carri & Cozzolino, 2011; De Vos, Grierson, Ackerley, & Miller, 2008; Magrane & Manfredi, 2009). Mutant SOD1 has been shown to localize to the inner membrane space and matrix of the mitochondria with the aid of its copper chaperone (CCS), which binds to Mia40. They then enter the mitochondria (depicted in **figure 3)** through the Erv1/Mia40 oxidative folding mechanism of import (Carri & Cozzolino, 2011). Once inside the mitochondria, mutant SOD1 accumulates in and forms oligomers (Deng et al., 2006; Ferri et al., 2006) (Furukawa, Fu, Deng, Siddique, & O'Halloran, 2006).

Figure 3 ETC and localization of SOD1 in mitochondria

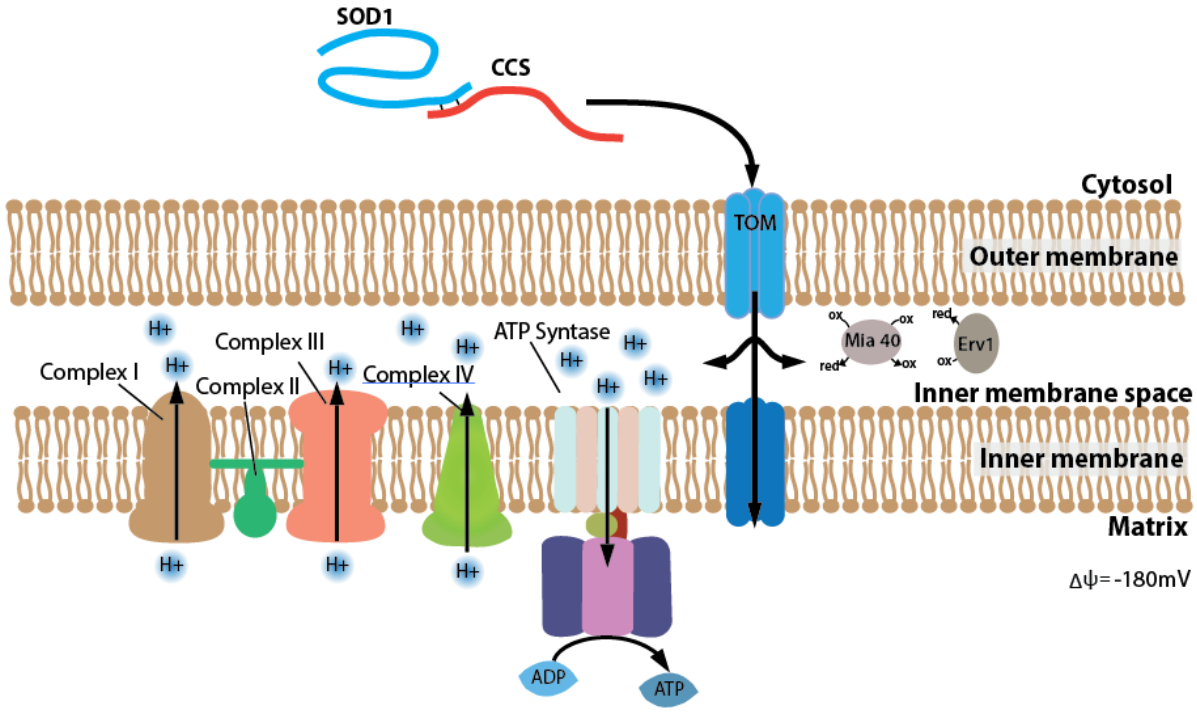


Figure 3 Wild type function of ETC and localization of SOD1 in mitochondria.

Protons are stored and allowed to build up in the inter membrane space while at the same time electrons are sent into the matrix where they contribute to a buildup of negative charge gradient thus establishing a negative potential in the matrix. This negative membrane potential then attracts protons as well as positively charged amino acids that are part of the localization signaling sequence for mitochondrial proteins. The movement of protons from the inner membrane space to the matrix is what drives the protein complex that produces ATP. As a consequence of this process a deadly byproduct called superoxide ($\mathbf{0_2^-}$) is produced, establishing mitochondria as the major producer of toxic reactive oxygen species. (Carri & Cozzolino, 2011).

Further, there are reports that mitochondrial association of PTEN-induced kinase (PINK1), a PD-linked serine/therine kinase, together with the activation of microtubule-associated protein 1 light chain 3 (LC3) occurring together are signs of mitochondrial autophagy activation and have been shown to be induced by overexpression of mutant SOD1 (Carri & Cozzolino, 2011; Ferri et al., 2006; Morimoto et al., 2010; Morimoto et al., 2007;

Pizzasegola et al., 2009). PINK1 itself has been implicated in mitophagy a cellular process of removing dysfunctional mitochondria. (Deas, Wood, & Plun-Favreau, 2011) This process may be activated as a result of disruption of mutant SOD1 to the electron transport chain (ETC) (Cozzolino et al., 2009) and can lead to mislocalization of mitochondria from the neural muscular junction as seen in **figure 4** (Nguyen, Garcia-Chacon, Barrett, Barrett, & David, 2009). Indeed, mitochondrial mislocalization in the axon has been seen in ALS and is suggested to be a cause of axonal degeneration (Han et al., 2012; Tsuda et al., 2008). Additionally, mutant SOD1 can aggregate in the matrix or inter membrane space and measures to remove or decrease aggregated mutant SOD1 in mitochondria has been shown to be effective at protecting the cell from mutant SOD1 induced toxicity (Carri & Cozzolino, 2011).

Figure 4. Neuromuscular junction deterioration

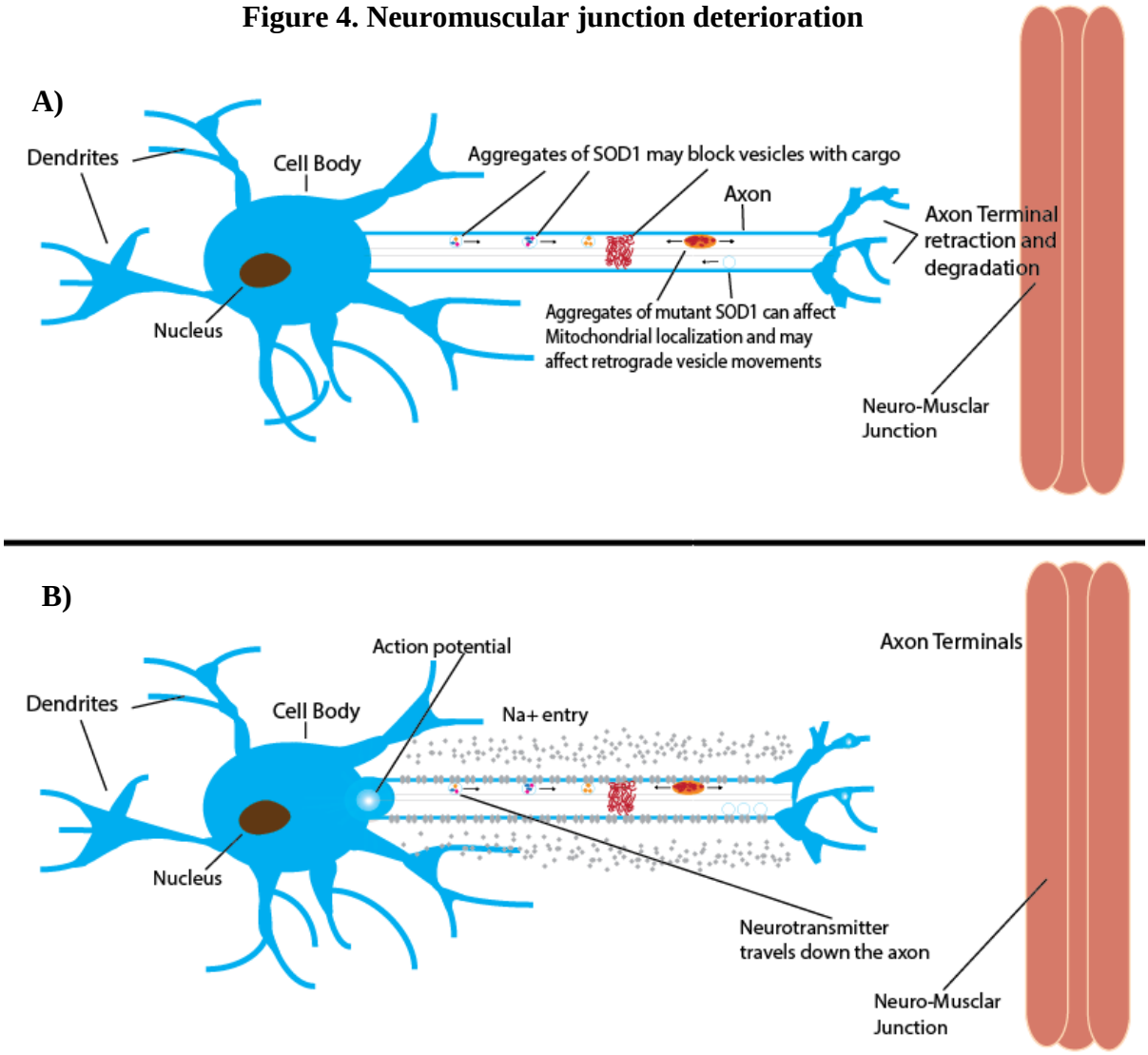


Figure 4 Aggregates of SOD1 can inhibit axonal travel and mitochondria localization.

Image (A) depicts aggregates of mutant SOD1 may inhibit vesicle travel and mitochondria localization to axon terminal. Thus contributing to axonal detachment and retraction from the neuromuscular junction (NMJ)>\.

Image (B) shows an action potential which may be ineffective because of axon retraction from the neuromuscular junction, exacerbating the degeneration process.

Aberrant Calcium Signaling in ALS

One study has shown that human mutant SOD1-G93A in SH-SY5Y cells showed a decreased ability to clear calcium from the cytosol to intracellular storage sites, such as the mitochondria and endoplasmic reticulum, or to the extracellular space. It has been postulated that this inability to clear calcium from the cytosol could account for the lack of calcium oscillation seen in these mutant SOD1 transfected cells (Jaiswal et al., 2009). This leads to a calcium buildup as a result of a storage and handling deficit, which is seen as an increase in cytosolic calcium concentration a hallmark of excitotoxicity and deadly to the neuron.

It has been shown with thapsigargin treatment, which induces endoplasmic reticulum (ER) stress, of SH-SY5Y cells transfected with G93A mutation of hSOD1 that selective release of calcium from the (ER) only slightly increases cytosolic calcium concentration over the similarly tested wild-type hSOD1 expressing cells. This indicates that dysfunction of stores of calcium in the ER only slightly contributes to motor neuron dysfunction and confirms the mitochondria as major players with regard to calcium accumulation and dysfunction (Jaiswal et al., 2009). When mitochondrial ability to store calcium becomes dysfunctional as seen in G93A mutant SOD1 transfected cells, neurons are then subject to cytosolic calcium accumulation. This then puts greater stress on the neuron and leaves it susceptible to calcium mediated cell death pathways resulting in neruodegeneration (Jaiswal et al., 2009).

Cytosolic increases in calcium concentration, which are seen in ALS, may be the result of oxidative stress to mitochondria (see below) and can lead to an increase in the production of nitric oxide and peroxynitrite both of which could be deadly to the cell (Rao & Weiss, 2004; Raoul et al., 2002).

Oxidative Stress

As noted, SOD1 is the major cytosolic protein to prevent oxidative damage and reduce superoxide leakage form mitochondria. It has been proposed that mutant SOD1 could reverse its protective catalytic activity, instead of scavenging and dismutation of superoxide (0_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), it might be able revert to produce toxic superoxide (Beckman et al., 2001; S. I. Liochev & Fridovich, 2000; Stefan I. Liochev & Fridovich, 2003). This could be the result of mutations that leave SOD1 defective in binding co-factor Zinc (Stefan I. Liochev & Fridovich, 2003). In such a state SOD1 could actually take electrons from other cellular antioxidants and then donate them to molecular oxygen, thus producing superoxide, making SOD1 the source of the oxidative stress(Beckman et al., 2001). The end result is postulated to be the production of peroxynitrite (ONOO) from superoxide and nitric oxide (Beckman & Koppenol, 1996). This would cause the production of tyrosine nitration by reaction with peroxynitrite. It is suggested that the critical protein affected by nitro-tyrosine residues is microtublues, thus interfering with their ability to polymerize, which in turn could inhibit proper subcellular trafficking and localization of mitochondria and neurotransmitter vesicle travel. (Beckman et al., 2001) However, the clinical and experimental evidence does not support that hypothesis (Boillee, Vande Velde, et al., 2006; Bruijn et al., 2004; Stefan I. Liochev & Fridovich, 2003).

Similar hypothesis regarding aberrant neuronal chemistry caused by the inability of mutant SOD1 to complex with Cu and Zn leaving excess cytosolic Cu and Zn ions to run amuck in the neurons have also not been supported by experimental and clinical data(Boillee, Vande Velde, et al., 2006). Additionally, mice transfected with $SOD1^{quad}$ mutants, which have completely null copper-binding sites, did not show signs of any of the above mentioned

aberrant chemistry that would result in toxic superoxide or nitration species nor is disease course altered (Wang et al., 2003).

ALS as a result of mutant SOD1 is not related to superoxide buildup and thus not a consequence of oxidative damage or stress cause by superoxide. Individual mutations have been documented to be throughout the SOD1 gene and yet all cause the same neuronal degeneration with minimal differentiation. Sporadic and familial ALS, along with other neurodegenerative diseases have signs consistent with a deficit in protein homeostasis, such as aggregation and mislocalization of mutated and wild type RNA regulatory proteins, like TDP-43. Thus, questions regarding aberrant protein accumulation and aggregation must be solved in order to comprehensively address neurodegeneration. Elucidating genes which are involved in the regulation of the aggregate formation process could contribute to a more complete understanding of neurodegeneration as a whole.

CHAPTER THREE

The Goal of the Project and the Research Design

The goal of this project

The goal of this project is to test the feasibility of using *Drosophila* model system to study the regulation of aggregates formed by the human SOD1 (hSOD1) protein. Abnormal protein aggregation is a prominent pathological hallmark of ALS. Their formation, by the ALS-associated disease proteins such as mutant SOD1, TDP43, FUS1, has been linked to the dysfunction and degeneration of motor neurons during disease pathogenesis. In particular, mutations in the small SOD1 gene have been established to be the cause of 20% of familial ALS. The long-term goal of the project is to systematically identify and analyze the regulators of aggregates formation by the mutated human SOD1 and other ALS-associated protein. This will enable further dissection of the molecular networks that regulate intracellular protein mis-folding and clearance in general, as well as provide potential targets for therapeutic interventions against this fatal disease.

***Drosophila*, an excellent model organism to study human diseases**

Drosophila, one of the best-studied genetic organisms, has emerged as an excellent model system to study human brain degenerative diseases. Although a simple model organism, the fruit fly offers several important advantages. For example, as a relatively simple invertebrate, it is small in size, easy to maintain and care for, and can be raised in large quantities at low cost. Second, it has high fecundity and short generation time, which is usually not longer than 13 days from egg to reproductive-competent mature adult. These features make *Drosophila* an ideal system to be subjected to large-scale and detailed genetic analysis. In fact, over the last one hundred years, the fruit fly has been one of the most extensively characterized and genetically manipulated multi-cellular organisms. In that time

a large arsenal of sophisticated experimental tools have been developed, such as the easily-applied transgenic techniques and targeted mutagenesis. Utilized by many and important to this study is the UAS-Gal4 binary expression system for targeted overexpression or knockdown of a target gene. Further, clonal mosaic systems for analyzing lethal mutations and differentiating cell-autonomous effect. Along with the recently developed genome-wide dsRNA libraries and RNAi transgenic lines for convenient knock-down of every single gene in the fly genome in both cultured cells and the whole animals. Moreover, *Drosophila* has a well-documented developmental process as well as extensively studied and highly-conserved signaling pathways. Notably, many important signaling pathways such as Wingless/Wnt, Notch, Hedgehog and Toll/innate immunity, were first discovered and elucidated in the fruit flies and still bear their original colorful names from this simple species. Furthermore, the fly genome also contains homologs of many human neurodegenerative disease genes, such as PD genes Parkin and Pink1, HD gene Huntingtin, AD gene App and Tau and ALS gene SOD1 and TDP43. Importantly to this study, the fruit fly also has a relatively simple yet conserved nervous system that bears many similarities with that in humans. These include, but are not limited to, diverse neuronal cell types such as sensory and motor neurons, the existence of glia cells, as well as the similar neuronal transmitters such as histamine, dopamine and glutamate signaling. Over the last decade, many human brain degenerative diseases including AD, PD and HD have been successfully modeled in the fruit fly (Bilen & Bonini, 2005; Marsh & Thompson, 2006). Finally, of particular relevance to this study, *Drosophila* and human copper chaperons have been shown to be highly conserved and functionally interchangeable (Hua, Georgiev, Schaffner, & Steiger, 2010). Thus, wild type

and mutant hSOD1 expressed in *Drosophila* is likely to complex with copper as it would in mammalians.

Experimental Design

Cell line- and whole animal-based ALS/SOD1 models in *Drosophila*

To study the regulation of the aggregate formation process by mutant SOD1, we have decided to develop both cell line- and whole animal-based human SOD1 models in *Drosophila*. The cell line-based model allows quick analysis of hSOD1 protein expression and aggregation potential in vitro. Further, the cell-based model can very easily scaled up such as to 384-well plate format that can be used for to high-throughput cell-based screens such as RNAi or bioactive compounds. The whole-animal based hSOD1 model enables us to analyze the *in vivo* effect of SOD1 in their ability to develop aggregates and induce toxicity in different tissues and cell types such as neuron verse glia. If both models are successful, we can further establish stable cell line models that express mutant human SOD1. Such a cell model is amenable to high-throughput screens such as genome-wide RNAi to quickly investigate every gene in the fly genome for their role in regulating mutant hSOD1 aggregation and toxicity. Extending this study to a screen for chemical compounds that could directly yield potential drug candidates would be quick and simple. Subsequently, the whole-animal models can facilitate *in vivo* validation of any identified hits from the cell-based screens.

Selection of hSOD1 mutations for the study

For mutant hSOD1, we will focus on the three most-characterized ALS mutations: A4V, G85R and G93A. Human SOD1 mutation A4V it the most prominent mutation of fALS associated SOD1, and has been shown to be particularly unstable and aggressive at forming aggregates ("ALS association," 2013; Boillee, Vande Velde, et al., 2006). However, it remains catalytically active as does the well characterized G93A mutation which was used to establish the first hSOD1 associated ALS mouse model (Bruijn et al., 2004; Spalloni et al., 2004). Furthermore like mutation A4V of SOD1 the G85R is also aggressive at forming aggregates, but is catalytically dysfunctional (Boillee, Vande Velde, et al., 2006; Watson, Lagow, Xu, Zhang, & Bonini, 2008). The mutations focused on for this study provide a good representative sample of the over 150 ALS associated hSOD1 mutations. As the control, we will include the wild type human SOD1 (WT-hSOD1) in all the studies to eliminate background effect and false positives.

Tagging with fluorescent reporters for convenient monitoring of SOD1 protein expression and aggregate formation

To facilitate convenient visualization of gene expression and subsequent aggregate formation, both wild type (WT) and mutant hSOD1 will be fused to either eGFP or mCherry fluorescent proteins with a short flexible linker mainly composed of small amino acid glycine and serine (GS). The use of the two different fluorescent reporters allows easy distinction of mutant hSOD1 and control WT- hSOD1 proteins co-expressed in the same cell. For example, in the cell-based model, plasmids containing wild-type hSOD1 fused to mCherry reporter will be co-transfected with mutant hSOD1 with eGFP reporter. With this co-transfection scheme, the presence of red-colored WT-hSOD1 will serve as an internal control

for the protein expression level of transfected DNA, which is a critical factor in protein aggregation. More importantly, it also allows clear distinction of whether the effect on aggregate formation from a study is specific for mutant hSOD1, which will be the targets of interest for follow up study. Or are the observed effects on aggregate formation non-specific and affect both mutant and WT-hSOD1. Such an observation would likely mean we have a false positive due to background effect and should be disregarded.

Cell-based model

We have selected the pMK33 vector as cloning template mainly for the following reasons. First, pMK33 contains a copper-inducible metallothionein promoter, which allows for temporal control of SOD1 expression by the addition or removal of copper from the medium. This is preferred over constitutive expression such as by actin promoter which might not be feasible for potentially toxic proteins such as SOD1. In addition, insoluble aggregates may be impervious to regulation, thus for an accurate assessment of a gene's ability to regulate aggregate formation, it must be knocked down significantly before aggregates form. Thus, in a RNAi or compound screen, before inducing hSOD1 expression, we are able to first incubate the cells in the reagents such as dsRNA. This can be done for a significant amount of time so as to efficiently knockdown the expression level of the target gene. Moreover, it is important to consider that eGFP can take hours to fold completely and fluoresce(Chudakov, Matz, Lukyanov, & Lukyanov, 2010). Lastly, this vector contains a hygromycin resistance gene, which will allow for convenient establishment of stably transfected cell-line through hygromycin selection.

Animal-based model

For the animal-based model**,** we will use the pUAST transgenic vector as cloning template, which can be conveniently combined with the Gal4 system to achieve targeted expression of studied genes in almost any temporal- and spatially-controlled manner in the transgenic flies diagramed in **figure 5**.

Figure 5. The UAS-GAL4 binary expression system

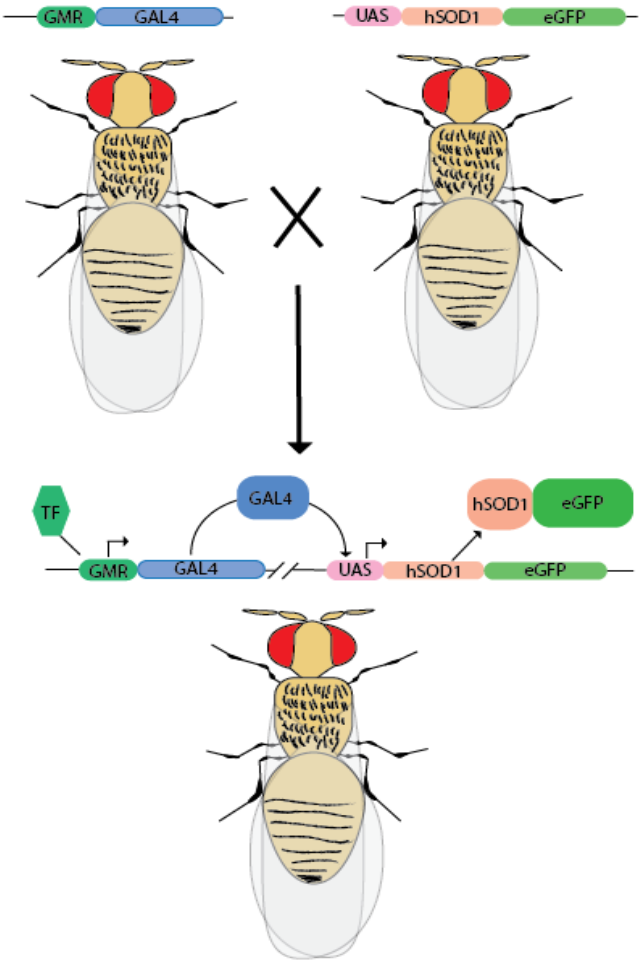


Figure 5 The GAL 4-UAS binary expression system in *Drosophila* is originally derived from *S. cerevisiae.*

In the yeast, in the presence of galactose as a primary source of food, yeast will express a family of genes under the upstream activating (UAS) response element, which is recognized and activated by the GAL4 transcription factor. This expression paradigm is exploited in *Drosophila, using tissue-specific promoter to drive Gal4 expression (such as eye-specific GMR promoter) from one particular Gal4 line, which in turn* direct tissue-specific expression of desired gene cloned downstream of the UAS elements in another transgene.

The strategy for this system is as the following: the gene of interest is placed under the UAS response element promoter site (normally in the vectors pUAST for expression in somatic tissues or pUASP for expression in germ lines) and inserted into the fly genome at random. Expression of the GAL4 transcription factor is placed under the control of a *Drosophila* tissue specific response element. For example, GMR is an eye specific *Drosophila* response element recognized by a transcription factor that is primarily expressed in the eye, thus the GAL4 gene downstream of this response element will be expressed specifically in the eye. In the presence of a UAS-transgene, it can then find the correct UAS site and express the gene downstream of the UAS site with tissue specificity. In this way the expression of a transgenic gene can be accurately controlled. Importantly, a vast number of tissue-, cell-type and-or temporal-specific Gal4 lines have been established in *Drosophila*. Which are easily available from several national and international *Drosophila* stock centers. Important to this study are the motor neuron specific D42-Gal4, pan-neuronal specific Elav- and Appl-Gal4, the latter is more specific for expression in adult stage on, and glial-specific Repo and GCM-Gal4 lines. Thus, the employment of pUAST enables us to easily target hSOD1 transgene expression to any tissue of interest and examine their effects.

Rationale

Extensive studies have demonstrated that aggregates formation are tightly linked to pathogenesis of ALS and other neuronal degenerative diseases. In ALS, mutant SOD1 proteins are known to be more prone to mis-folding and form protein aggregates. For example, in previous mammalian cell-based ALS models, in both neuron-like non-dividing NSC34 cells and dividing COS1 cells of monkey origin, GFP-tagged mutant human SOD1 including mutations A4V, G85R and G93A, but not wild type hSOD1, developed aggregates

(Corcoran, Mitchison, & Liu, 2004; Matsumoto, Stojanovic, Holmberg, Kim, & Morimoto, 2005). Thus, a detailed understanding of how cells respond to and regulate aggregates formation by mutant SOD1 will help elucidate the molecular mechanisms that control protein mis-folding and aggregation, and also potentially identify therapeutic targets for treating this deadly neurodegenerative disorder.

Nevertheless, to our knowledge, except in *Caenorhabditis elegans*, few such systematic studies have been conducted in multicellular model organisms. In a reported *C. elegans* study, a genome-wide RNAi screen was carried out in a worm ALS model with pan-neuronal expression of human G85R SOD1 mutant tagged with a yellow fluorescent fusion (YFP) reproter, from which more than 80 dsRNA hits that caused enhanced aggregates and diminished locomotion of the animal were isolated (Wang et al., 2009). We thus hypothesize that similar studies, in additional model organisms that include more strigent controls to screen for both suppressors and enhancers of mutant SOD1aggregates, will not only provide a more comprehensive picture of the aggregation regulators in the cell, but also help narrow down the conserved central players that control mutant SOD1 misfolding and accumulation.

Previous studies have demonstrated that mutant human SOD1 can also form protein aggregates and induce animal toxicity in *Drosophila*, another well-established model organism (Wang et al., 2009; Watson et al., 2008). Accordingly , we have planned to use the tried and trusted versatile tools in this excellent animal model to systematic study the regulation of aggregate formation by ALS associated human SOD1mutants. Our study is an important and logical next step, as most signlaing pathways are highly conserved between the fly and human, while a screen in the fly will be more cost effective and less time consuming than that in a mouse model. In addition, establishing, in parrallel, a whole-animal and also a

cell-based ALS model in *Drosophila* will allow for more extensive controls and easy validation of observations and results found in either model. With the previous experience in aggregates-centered screens on Huntington's disease and resources that are readily available in our lab, we have expected that this ALS project will allow us to thoroughly and accurately evaluate the feasibility of employing *Drosophila* model to study the regulators of aggregates by mutant hSOD1, laying important foundation for more comprehensive studies in the future.

CHAPTER FOUR

Molecular Cloning, Methods and Experimental Procedures

Molecular Cloning to Generate Fluorescent-tagged hSOD1 Fusion Protein

The four genetic variations of human SOD1 used in the project are A4V, G85R, and G93A and wild type (WT) control, as discussed in the previous chapter. The cDNA clones containing each of the mutations and wild type hSOD1 were generously provided by Dr. Daryl Bosco (University of Massachusettes Medical School, Worcester). The sequences of each SOD1 construct were verified by DNA sequencing after every cloning step.

Each of the four genetic variations of hSOD1 will be fused in frame enhanced green fluorescent protein (eGFP) and to the red fluorescent protein; mCherry, a derivative of eGFP, thus a total of 8 fusion combinations.

pMK33-hSOD1 constructs for cell line-based models

The pMK33 vector is 8.5kbp in size and contains multiple restriction sites. For this project we utilized XhoI and SpeI, which correspondingly match an Xho1 C-terminal restriction site on PCR amplified hSOD1 cDNA and a SpeI site at the N-terminal of the PCR amplified eGFP and mCherry sequences (See details below in **Summary of cloning procedures**).

pUAST-hSOD1 constructs for transgenic animals

To generate transgenic fly models for SOD1, we used the transgenic vector pUAST, which is 9Kbp in size by itself. It contains unique XhoI and XbaI cloning sites for the same aforementioned hSOD1 gene construct insertions (Also see details below in **Summary of cloning procedures)**. The vector also contains a mini-*white* gene, which restores the

Drosophila red eye phenotype in the otherwise white eye color w^{1118} flies, so as to allow easily determination of the success of transgenic events. It also allows for the use of the UAS-GAL4 binary expression system to control tissue specific ectopic gene expression. Amplified and digested hSOD1 will be fused in frame at its N-terminus, through a PCR-introduced AgeI restriction site on the GS linker (see below), with the C-terminus of the chosen fluorescent marker.

Thus**,** as there are 8 different combinations of hSOD1 varieties with eGFP and mCherry fusions. In order to establish both the cell line- and the whole animal-based models to study regulators of aggregate formation by ALS-associated human SOD1 (hSOD1) mutants, we would need to clone them into the selected vectors. A pUAST vector was utilized to establish the whole animal-based model, and the pMK33 vector utilized to establish the cell line-based model, thus for a total of 16 constructs, as summarized in **Table 1** of the results section.

Summary of cloning procedures

The cloning strategy involved seven steps. (1) Both wild type and mutant hSOD1 as well as the fluorescent reporters eGFP and mCherry were amplified by PCR, which used primers with added restriction enzyme sites such as XhoI or AgeI at both ends to facilitate cloning. A GS linker sequence encoding a short stretch of small amino acid glycine (G) and serine (S) was also added to one end of the primers, so as to fuse the SOD1 open reading frame with the eGFP or mCherry reporter; (2) The amplified SOD1 and eGFP/mCherry were cut with restriction enzymes and then purified with a gel extraction kit from Qiagen and stored at -20C until further use; (3) The cloning vector pUAST (for generating transgenic

flies) and pMK33 (cell transfection) were similarly cut with matching restriction enzymes and gel purified; (4) Next, a triple ligation of the digested vector, hSOD1 and eGFP or mCherry fragments was performed with T4 ligase ; (5) These ligations were transformed into competent *E. coli* bacteria cells and grown on selection plates for 16 hours, and correct clones were identified by PCR; (6) Extraction and purification of the plasmids was accomplished with Qiagen mini-prep kits; (7) Plasmid DNA were diagnosed with digestion by restriction enzymes and separation by agarose gel. The correct plasmids were further verified by Sanger DNA sequencing (Lone Star labs).

List of primers:

SOD1 forward primer: 5'-CTCGAGGAATTCCTAGAAATAATTTTGTTTAA-'3

SOD1 reverse primer with GS linker:

5'-GTGTAATTGGGATCGCCCAAGGCGGATCCTCCGGAGGTACCGGT-3'

eGFP forward primer: 5'-GTGAGCAAGGGCGAGGAG-3'

eGFP reverse primer: 5'-CCCTGAACCTGAAACATAACTAGT-3'

mCherry forward primer: 5'-ATAACCGGTGTGAGCAAGGCGAGGAGG-3'

mCherry reverse primer: 5'- GGCTGATTATGATCTAGAGTCGCG-3'

Summary of Protocols

PCR protocol: Either Taq or Phusion PCR polymerase was employed with the appropriate buffer. In general a master mix of PCR reagents was made and included in this order: deionize water, 10x or 5x buffer, dNTP at 200μM each, 0.5 μM of primers, 1μl of polymerase and 50 ng/μl of template DNA. A final volume of 25μl per PCR reaction, the reaction cycle included three stages, the first was denaturing at 98°C for 30 seconds and the second stage was 25-30 cycles which included a denaturation step at 98°C for 10 seconds, anneal step at 56°C for 23 seconds and extension step at 72°C for 1 minute and 20 seconds and then a final stage of extension at 72°C for 5 minutes. The final product was then purified using a Qiagen DNA purification Kit. Although, some steps were not necessary and no modifications were made to the standard protocol.

Restriction enzyme: All restriction enzymes used in the project required a buffer (NEB buffer number 4) containing 50mM Potassium Acetate, 20mM Tris-acetate, 10mM Magnesium Acetate, 1mM DTT and a pH of 7.9 at 25°C. A master mix was used containing 2 μl of New England Biolabs(NEB) buffer number 4, 0.3μl of each restriction enzyme, 1ng of template and 20μl deionized water per reaction. After digestion the entire reaction mixture was loaded into a 2% agarose gel with ethidium bromide to highlight DNA under ultra violet light and separated with 135mv for 12 minutes. The gel was then visualized under UV light and the DNA bands of the correct size were excised from the gel and place into eppendorf tubes. The DNA was then extracted from the agarose gel following the protocols from the Qiagen Gel Purification Kit which.

DNA Ligation protocol: T4 DNA ligase was used for all ligations with a buffer containing 50mM Tris-HCl, 10mM $MgCl_2$, 1mM ATP, 10mM DTT, at a pH of 7.5 at 25°C. The components for a 20ul reaction were; 10x T4 DNA ligase buffer at 2µl, vector DNA 50ng and insert DNA 50ng, Nuclease-free water up to 20µl and 1µl of T4 DNA ligase. The ligation was then place in an 18°C incubator overnight but can also be performed at 25°C for one hour.

***E. Coli* Transformation protocol:** Transformation is a common molecular biology technique employed to select and amplify the newly generated DNA constructs after ligation of digested insert and vector DNA fragments. This technique exploits the natural ability of some bacteria, specifically *E.Coli* used in this study, to accept and amplify foreign plasmids under certain treatment. In my study, I used a standard transformation protocol involved 7 basic steps; (1) add ~11 µl of ligation sample to ~170µl of competent *E. Coli.* (2) Ice for 20 mins to allow bacteria to settle and plasmids to settle on bacteria outer membrane surface. (3) Heat shock for no longer than 90 seconds at 42°C. This will open pores in the bacteria membrane and allow plasmids to enter. (4) Immediately ice for 20 minutes to allow bacteria to rest and the pores formed to close. It is important not to disturb the bacteria during this time as they are in a very fragile state. (5) Add ~150µl of liquid broth and (6) incubate with constant agitation at 37°C for 40 to 50 minutes. (7) Spread entire volume on an agar plate that contains antibiotic. The plasmids used in this project contained an ampicillin resistance gene so that antibiotic was incorporated into the mixture when making the plate.

Generating UAS-hSOD1-eGFP and UAS-hSOD1-mCherry transgenic flies

After sequence verification, the SOD1-eGFP or SOD1-mCherry constructs in pUAST vector were microinjected into w^{1118} fly embryos following standard protocol. The flies were allowed to mature and then crossed with w^{1118} virgins or males, and the resulting F1 progenies was screened for restoration of red eye color from the white eye flies of the parental w^{1118} background, which indicated the success of transgenic events. The independent transgenic lines were then crossed to double balancer flies to map the chromosomal location of the transgenic inserts and also to balance the transgene in the genome. Double balancer flies contain chromosomes that have been inverted and rearranged so that homologous recombination cannot occur. This will help stabilize the inserted gene so it is not loss through generations of mating. The balancer flies employed in this project were double balancer flies that contained second and third chromosome balancers. They were distinguished by two phenotypes and two markers. For the second chromosome the phenotype was a curly wing called *cyo* and a marker called *sp* that is recognized by a change in follicle grouping near the first leg on both sides. The third chromosome phenotype was a change in bristle length on dorsal side of the thorax and it is called $TM6_C$ and a marker that is seen as a change in eye shape and is called dropper (*Dr*).

***Drosophila* Genetics:**

A total of 4 transgenic *UAS*-hSOD1-wild type fused to eGFP lines were tested during this project along with 3 lines for *UAS*-hSOD1 mutation A4V and G93A. Two lines of transgenic *UAS*-hSOD1-G85R fused to eGFP and two lines fused to mCherry were also tested. The transgene were mapped and their chromosomal locations were determined according to standard procedures using balancer stocks. For transgenic lines inserted on the second chromosome, they were subsequently balanced with SM5a, *Cyo* ; those on the third

chromosome were balanced with *TM6c balancers*. The GAL4 lines selected to direct expression of the hSOD1 transgenic flies in this project were GMR-GAL4 (eye specific), D42 -GAL4 (motor neuron-specific) and GCM-GAL4 (glia cell during early development).

Genotypes for GMR-GAL4 drivers:

The genotype of the progeny transgenic flies for *UAS*-hSOD1-WT-eGFP on the third chromosome after cross with GMR-Gal4 was: *GMR-GAL4/+; UAS-hSOD1-WT-eGFP /+; +/+*. The genotype of the progeny transgenic flies for *UAS*-hSOD1-WT-eGFP on the second chromosome after cross with GMR was: *GMR-GAL4/ UAS-hSOD1-WT-eGFP; + /+; +/+*.

The genotype of the transgenic, *UAS*-hSOD1-A4V-eGFP on the third chromosome, after cross with GMR-Gal4 was: *GMR-GAL4/+; UAS-hSOD1-A4V-eGFP /+; +/+*. For the second chromosome it was *GMR-GAL4/ UAS-hSOD1- A4V -eGFP; + /+; +/+*.

The genotype of the transgenic hSOD1-G85R-eGFP on the third chromosome, after cross with GMR-Gal4 was: *GMR-GAL4/+; UAS- hSOD1-G85R-eGFP /+; +/+*. For the second chromosome it was *GMR-GAL4/ UAS-hSOD1- G85R -eGFP; + /+; +/+*.

The genotype of the transgenic *UAS*-hSOD1-G85R-wCherry on the third chromosome, after cross with GMR-Gal4 was: *GMR-GAL4/+; UAS-hSOD1-G85R-mCherry /+; +/+*. For the second chromosome it was *GMR-GAL4/ UAS-hSOD1- G85R-mCherry; + /+; +/+*.

The genotype of the transgenic *UAS*-hSOD1-G93A-eGFP on the third chromosome, after cross with GMR-Gal4 was: *GMR-GAL4/+; UAS-hSOD1-G93A-eGFP /+; +/+*. For the second chromosome it was *GMR-GAL4/ UAS-hSOD1- G93A -eGFP; + /+; +/+*.

Genotypes for using motor neuron-specific D42-GAL4 drivers:

The genotype of the progeny transgenic flies for *UAS*-hSOD1-WT-eGFP on the third chromosome after cross with motor neuron-specific D42-Gal4 line was: D42-Gal4 */+; UAS-hSOD1-WT-eGFP /+; +/+*. The genotype of the progeny transgenic flies for *UAS*-hSOD1-WT-eGFP on the second chromosome after cross with D42-Gal4 line was: D42-Gal4*/ UAS-hSOD1-WT-eGFP; + /+; +/+*.

The genotype of the transgenic, *UAS*-hSOD1-A4V-eGFP on the third chromosome, after cross with D42-Gal4 was: D42-Gal4*/+; UAS-hSOD1-A4V-eGFP /+; +/+*. For the second chromosome it was D42-Gal4*/ UAS-hSOD1- A4V -eGFP; + /+; +/+*.

The genotype of the transgenic hSOD1-G85R-eGFP on the third chromosome, after cross with D42-Gal4 line was: D42-Gal4*/+; UAS-hSOD1-G85R-eGFP /+; +/+*. For the second chromosome it was D42-Gal4*/ UAS-hSOD1- G85R -eGFP; + /+; +/+*.

The genotype of the transgenic *UAS*-hSOD1-G85R-wCherry on the third chromosome, after cross with motor neuron-specific D42-Gal4 was: *D42-GAL4/+; UAS- hSOD1-G85R-mCherry /+; +/+*. For the second chromosome it was *D42-GAL4/ UAS-hSOD1- G85R-mCherry; + /+; +/+*.

The genotype of the transgenic *UAS*-hSOD1-G93A-eGFP on the third chromosome, after cross with motor neuron-specific D42-Gal4 was: *D42-GAL4/+; UAS-hSOD1-G93A-eGFP /+; +/+*. For the second chromosome it was *D42-GAL4/ UAS-hSOD1- G93A -eGFP; + /+; +/+*.

Genotypes for GCM-GAL4 drivers:

The genotype of the progeny transgenic flies for *UAS*-hSOD1-WT-eGFP on the third chromosome after cross with GCM-GAL4 was: *GCM-GAL4/+; UAS-hSOD1-WT-eGFP /+; +/+*. The genotype of the progeny transgenic flies for *UAS*-hSOD1-WT-eGFP on the second chromosome after cross with GCM was: *GCM-GAL4/ UAS-hSOD1-WT-eGFP; + /+; +/+*.

The genotype of the transgenic *UAS*-hSOD1-G85R-eGFP on the third chromosome, after cross with GCM-GAL4 was: *GCM-GAL4/+; UAS-hSOD1-G85R-eGFP /+; +/+*. For the second chromosome it was *GCM-GAL4/ UAS-hSOD1- G85R -eGFP; + /+; +/+*.

The genotype of the transgenic hSOD1-G85R-wCherry on the third chromosome, after cross with GCM-GAL4 was: *GCM-GAL4/+; UAS-hSOD1-G85R-mCherry /+; +/+*. For the second chromosome it was *GCM-GAL4/ UAS-hSOD1- G85R-mCherry; + /+; +/+*.

Targeted overexpression of UAS-hSOD1-eGFP or UAS-hSOD1-mCherry using Gal4 system

The UAS-hSOD1-eGFP or UAS-hSOD1-mCherry transgenic flies were crossed to selected tissue-specific GAL4 drivers. Enabling us to check and verify correct transgene expression via western analysis, for phenotypic characterization and imaging documentation. For western analysis, 10 adult flies aged for 1 day expressing wild type hSOD1 and mutant G85R-hSOD1 fused to eGFP and mCherry, driven by the D42 (motor neuron) and GMR (eye specific) driver were homogenized in RIPO buffer. The samples were separated with electrophoresis of a polyacrylamide gel and then transferred to a nitrocellulose membrane.

To detect proteins present on the membrane, anti-eGFP and mCherry antibodies were used. The membrane was then imaged with a LI-COR imager on western blot membranes.

Dissection and staining of adult fly brain and ventral nerve cord

Adult flies expressing A4V- or G93A-hSOD1 mutation with eGFP tag were dissected in 1X PBS to remove their brain and ventral nerve cord. The samples were then fixed in 4% formaldehyde for 45 minutes, then stained with phlloidain and DAPI and placed on a slide to image with a fluorescent microscope.

Imaging of adult fly eyes

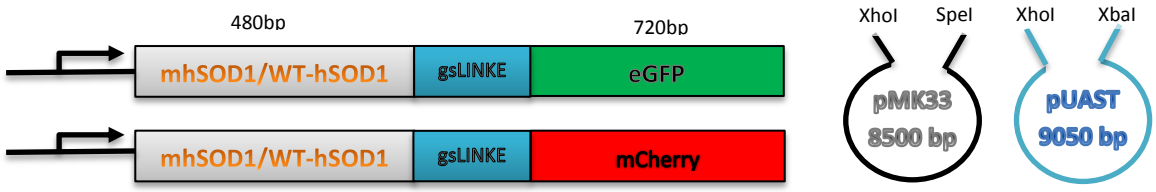
Flies expressing hSOD1 under the GMR-Gal4 eye specific driver were fixed to a slide with a fast drying clear liquid polymer and imaged with a fluorescent microscope. Images were taken under green or red UV illumination to visualize eGFP or mCherry signals and under bright light to image overall eye morphology.

CHAPTER FIVE

Cloning Results and Assessing hSOD1 Aggregation Formation in *Drosophila* Cell Lines

Table 1. Experiment design and molecular cloning status

(A



(B

Gene	Vectors Completed		Transfected and Expressing		Established transgenic line		Expressing	
	eGFP	mCherry	eGFP	mCherry	eGFP	mCherry	eGFP	mCherry
hSOD1-wt	✓	✓	✓	✓	✓	–	✓	–
hSOD1-A4V	✓	✓	✓	–	✓	✓	✓	–
hSOD1-G85R	✓	✓	✓	–	✓	✓	✓	✓
hSOD1-G93A	✓	✓	✓	–	✓	–	✓	–

Results of the Molecular Cloning

Importantly, from the study, all the 16 constructs were successfully generated and sequencing verified (see **Table 1).** The fly lines established thus far are displayed in **Table 2.** Thus, this work will establish the foundation for the ensuing cell- and whole animal-based studies.

Table 2: Summary of established transgenic fly lines

Fly Line	Chromosome location	Balancer	Expression Tested
UAS-hSOD1-WT-eGFP			
2-1-2	2	Cyo	✓
3-2-2	3	Tm6c	✓
1-1	3	Tm6c	✓
1-3	3	Tm6c	✓
UAS-hSOD1-A4V-eGFP			
2-1-2	3	Tm6c	–
2-2-1	3	Tm6c	✓
2-1-1	3	Tm6c	✓
2-3-1	3	Tm6c	✓
UAS-hSOD1-A4V-mCherry			
3-1	3	Tm6c	–
3-2	3	Tm6c	–
UAS-hSOD1-G93A-eGFP			
1-2-2	2	Cyo	✓
2-1-1	3	Tm6c	✓
2-1-2-2	3	Tm6c	–
2-1-2	3	Tm6c	–
3-1-4	3	Tm6c	–
3-1-4-1	3	Tm6c	✓
UAS-hSOD1-G85R-eGFP			
1-3	1	–	–
1-5	1	–	–
2-2	2	Cyo	–
4	2	Cyo	–
1-6	3	Tm6c	–
1-4	3	Tm6c	
1-2	3	Tm6c	✓
2-3-2	3	Tm6c	–
5	3	Tm6c	✓
UAS-hSOD1-G85R-mCherry			
1-2	2	Cyo	✓
1-3	2	Cyo	–
1-1	3	Tm6c	✓
2-2-1	3	Tm6c	–

To investigate establishing a cell-based model to study aggregate formation by mutant human SOD1 proteins, we first performed transient transfection assay to examine their expression from the established pMK33 constructs. Further, we were able to test whether the mutant hSOD1 proteins form robust aggregates in the cell. In the established pMK33 constructs, the expression of wild type and mutant hSOD1-eGFP or mCherry fusions were controlled under a copper-inducible metalothrenine protomor.

The S2 cells selected for this project are a macrophage lineage *Drosophila* cell line that is commonly utilized for fruit fly related *in vitro* studies. An advantaged of these cells is their relative robustness and hardiness when grown in S2 medium that has been supplimented with antibiotic and serum. For this study the cells were specifically cultured in Schneider S2 growth medium from invitrogen, which was supplamented with the antibiotics streptomycin and penicillian and with fetal bovine serum.

The established pMK33 constructs were transfected into *Drosophila* S2 cells. 48 hours after transfection, expression of fusion proteins were induced with aqueous copper sulfate solution at a final of concentration 250 mM.

Pilot transfection assays

In this study, S2 cells were first transfected with the pMK33 vector construct to assess proper expression of hSOD1 fused to eGFP under copper induction and they were assess visually for any obvious formation of mutant hSOD1-eGFP aggregates. For this test transfection, all four pMK33 constructs containing eGFP-tagged wild type or each of the three selected hSOD1 mutants were used separately. 48 hours after SOD1 expression was induced with copper at a final concentration of 250mM, the cells were visualized under a

fluorescent micrscope. Which revealed clear expression of each of the tested hSOD1-eGFP fusion, and the protein were predominantly localized to the cytosol, as expected. These observations suggested the successful transfection of the plasmids into the S2 cells and the proper induciton of SOD1 protein expression. However, after 24, 48 and 72 hours of induction, we did not observe obvious aggregation by either wild type or any of the three of hSOD1 mutations.

Co-transfection assays

A second transfection strategy involved co-transfection of wild type hSOD1 fused to mCherry and mutant G85R-hSOD1 fused to eGFP. The purpose of this stragey was to show a clear contrast between any presence of aggregated mutant G85R-hSOD1 (revealed by the green color of its eGFP tag) in the red background of a soulble wild type hSOD1 (revealed by its mCherry tag). In addition, there exist reports suggesting that co-expression of wild type and mutant hSOD1 may promote more aggressive aggregation and disease phenotype (Deng et al., 2006; Graffmo et al., 2013; Jonsson et al., 2004). However, when examined under fluorsecent microscopy 18 hours (**Figure 6**) or 24 hours (data not shown) after induciton of protein expression, we again did not obseve obvious aggregate formation in either of the tested conditions.

Figure 6. hSOD1 expression in *Drosophila* S2

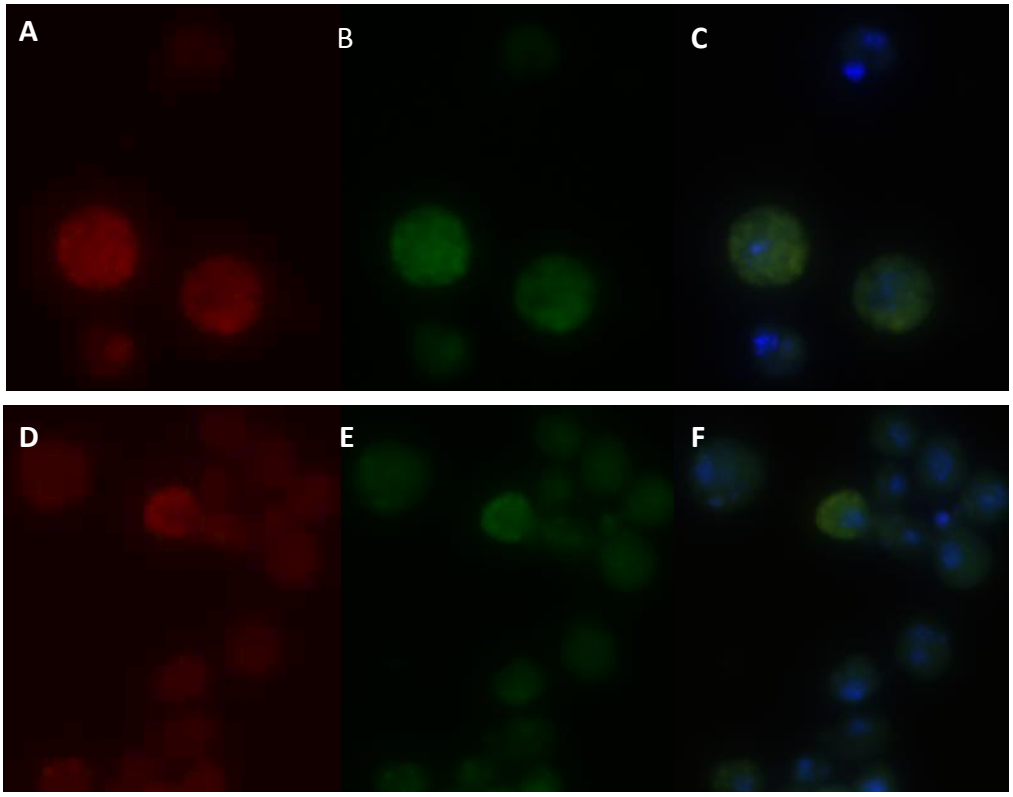


Figure 6. human SOD1 expression in *Drosophila* S2 cells.
S2 cells were co-transfected with wild type hSOD1-mCherry (red in **A** and **D**) and mutant G85R-hSOD1-eGFP (green in **B** and **E**). Images were taken 18 hours after induction of SOD1 expression, which were counter stained with DNA dye DAPI to reveal the cell nucleus (blue in **C** and **F**). Note that wild type and G85R mutant hSOD1 showed almost identical subcellular distribution pattern. Overlay of eGFP and DAPI channels (**C** and **F)** suggested that both wild type and mutant hSOD1 mainly localize to the cytoplasm. No obvious aggregation of SOD1 protein was observed. Imaged with 40X magnification with Zeiss Axio Fluorescent compound microscopy.

In summary, results from the cell-based transfection assays suggest that human SOD1, either as wild type or each of the tested mutant forms, do not form robust aggregates in Drosophila S2 cells. Due to this observation, we did not further pursue establishing stably transfected cell lines, as originally planned.

CHAPTER SIX

Studying hSOD1Aggregation and Toxicity in Transgenic Drosophila Models

In this chapter of the study, we applied the UAS-Gal4 system to overexpress human SOD1 transgenes in different *Drosophila* tissues. We then analyzed the ability of WT and mutant hSOD1to form aggregates *in vivo* and their effects on animal behavior and survival.

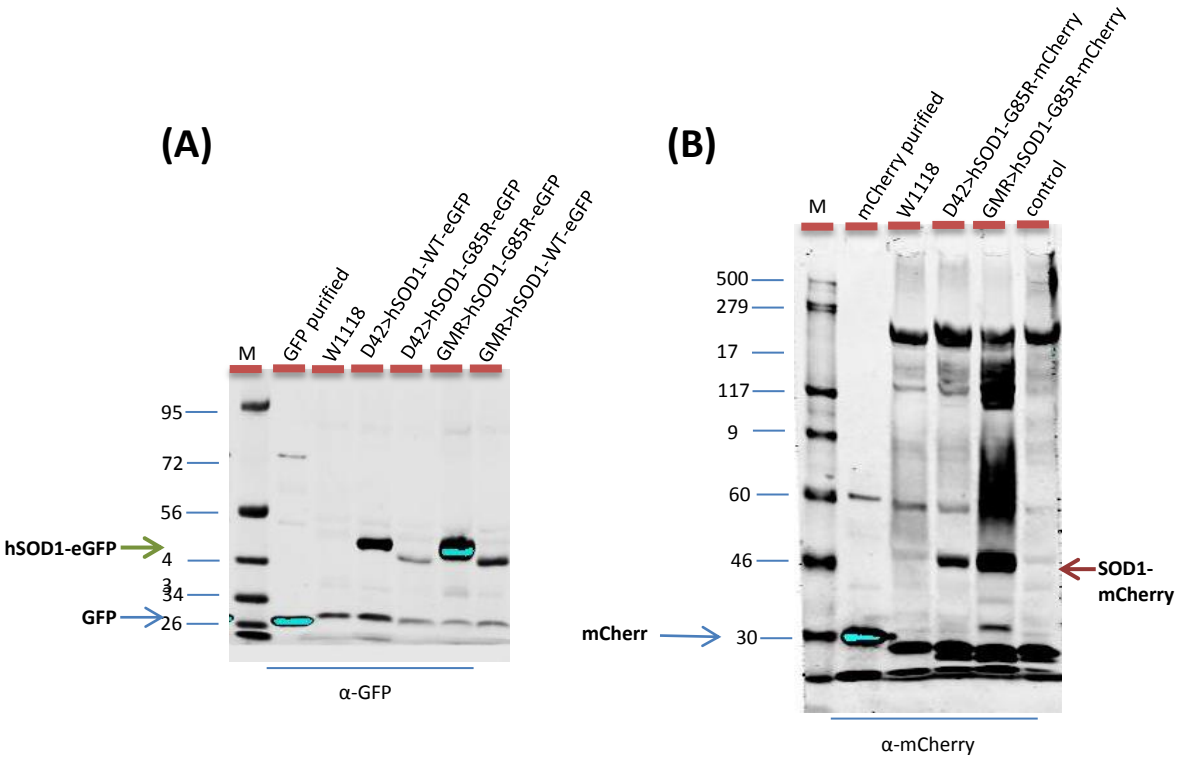
Confirmation of hSOD1 transgene expression in transgenic fly lines

To first confirm the proper expression of the hSOD1-eGFP or hSOD1-mCherry fusion proteins from the established UAS-hSOD1 transgenic flies, we crossed the wild type and mutants A4V, G85R and G93A SOD1 lines with D42 (motor neuron) and GMR (eye) GAL4 drivers. These GAL4 driver lines provide tissue specific control over transgenic hSOD1 expression. From each crosses, ten adult progenies containing both the GAL4 driver and UAS transgene were collected and homogenized to extract whole proteins, followed by western blot analysis to determine the success of protein expression from these transgenic lines.

Figure 7 shows the results of the flies expressing wild type (WTSOD1-eGFP) and mutant G85R (G85R-h SOD1-eGFP) hSOD1 fused to eGFP (Figure 2A), as well as samples of the same mutant fused to mCherry (G85R-hSOD1- mCherry, Figure 2B). Expression is controlled by either the motor neuron driver D42 GAL4 or the eye specific GMR driver. Expression of the fusion transgenes were detected with an anti-eGFP or anti-mCherry antibodies, respectively. hSOD1 encodes a small 16 kDa protein, whereas eGFP and mCherry alone encodes 26kDa and 30kDa proteins, respectively. Accordingly, the hSOD1-eGFP and hSOD1-mCherry fusion proteins should be at around 42 kDa and 46kDa sizes, respectively. Indeed, Western blot analysis confirmed the correct expression of these three

hSOD1 fusion proteins (WT-hSOD1-eGFP, G85R-hSOD1- eGFP and G85R-hSOD1-mCherry) at the predicted sizes, which were absent in the non-transgenic control flies (w^{1118} and control lane in 7A and 7B).

Figure 7. Western blot confirmation of hSOD1 expression in transgenic flies



GFP-tagged hSOD1 expression.

mCherry-tagged hSOD1 expression.

Figure 7. Western blot analysis of ectopic human SOD1 expression in transgenic flies.

Human SOD1 is a 16kda protein. mCherry and eGFP encode proteins of about 30kda and 26kda, respectively.

(A) Samples from flies with wild-type hSOD1 or mutant G85R-hSOD1 expression under the eye specific GMR-Gal4 and motor neuron-specific D42-GAL4 driver, as highlighted by the green arrow. Purified GFP alone was loaded in lane 2 as positive control (blue arrow). Sample from non-transgenic w^{1118} flies in lane 3 served as negative control.

(B) Samples from flies with mutant G85R-hSOD1 expression (highlighted by the red arrow) under the eye specific GMR-Gal4 or motor neuron-specific D42-GAL4 driver. Purified mCherry alone was loaded in lane 2 as control (blue arrow). Sample from non-transgenic w^{1118} flies in lane 3 served as negative control.

Quite interestingly, compared to WT-hSOD1-eGFP, the expression level of mutant G85R-hSOD1- eGFP was much lower in both D42 and GMR-Gal4 driven flies (Figure 2A). While the mutant G85R-hSOD1- eGFP protein also appeared to migrate slightly faster than the WT-hSOD1-eGFP in both cases, they have the same predicted protein size and differ only in one amino acid. Such a result, if held true, could suggest that the G85R mutation interferes with the stability of the expressed hSOD1 protein, rendering it unstable (i.e., reduced protein level) with potential conformational change (i.e., faster migration in the SDS-PAGE gel). Further studies are needed to verify the above observation, such as by using more independent UAS-hSOD1 transgenic lines for all three hSOD1 mutations and additional GAL4 drivers. Another approach would be applying the recently developed attP targeted insert technique to direct both wild type and mutant hSOD1 to the same chromosome location in the fly genome, thus eliminating the potential positional effect on gene expression levels of different transgenic lines.

Eye-specific over-expression of hSOD1 transgenes

After confirming the correct expression of SOD1 fusion proteins from the transgenic lies, we first examine the effect of their overexpression in the fly eye by using the eye-specific GMR-GAL4 driver. *Drosophila*'s eye is a popular tissue to characterize a gene's function and is also ideal for analyzing the potential neuronal toxicity of a studied protein. Adult *Drosophila* has a compound eye, composed of about 800 single units called ommatidium. Each ommatidium consists of neuronal bristles and eight photoreceptor cells, in addition to other non-neuronal accessory cells such as cone and pigment cells. In the normal adult eye, these ~800 eye units are patterned in a very regular and highly structured repetitive pattern, thus any mild effect on eye development will be magnified and easily

detected. Moreover, because it is a sensitive neuronal tissue that is dispensable for animal viability, it is ideal to monitor the neurodegenerative consequences of a manipulated gene on this neuronal tissue over an extended period of time. For example, gradual damage to the eye cells has been often shown to present as an easily recognizable loss of red eye color, due to the loss of internal pigment cells. Important to this study, as the eye is transparent, if the fluorescent-tagged mutant hSOD1 protein forms aggregates in the eye cells, they can be easily visualized directly under the fluorescent dissection microscope. This can be performed without further dissection, as has been shown for aggregates-forming mutant Huntington protein (Zhang, Binari, Zhou, & Perrimon, 2010), thus greatly facilitating the monitoring for aggregates formation inside the eye.

Figures 8-10 shows the adult *Drosophila*'s eyes at differt ages expressing fluorescent-tagged wild type and mutant hSOD1 from the transgenic flies, driven by the eye-specific GMR-Gal4 driver.

Figure 8 consists of images of eyes of 30-day-old adult flies expressing wild type hSOD1 and each of the mutant hSOD1 tagged with eGFP, along with eyes expressing mutant G85R-hSOD1 with mCherry tag. When examined under bright field microscopy, all of these eyes have regular well-patterned morphology, suggesting that overexpression of wild type or either of the hSOD1 mutants does not affect normal eye development. Notably, there is no clear loss of eye pigmentation in any of the hSOD1-expressing flies, even at age of day 70 (**Figure 9** for wild type and G85R-SOD1 mutation, the same was seen for A4V and G93A SOD1 mutants (data not shown). Indicating that there is no significant cellular degeneration in these hSOD1-expressing flies, which further suggest that overexpressed hSOD1, either wild type or any of the mutant forms, are well-tolerated by *Drosophila* eye cells.

Figure 8. hSOD1 expression under eye-specific GMR-Gal4 driver

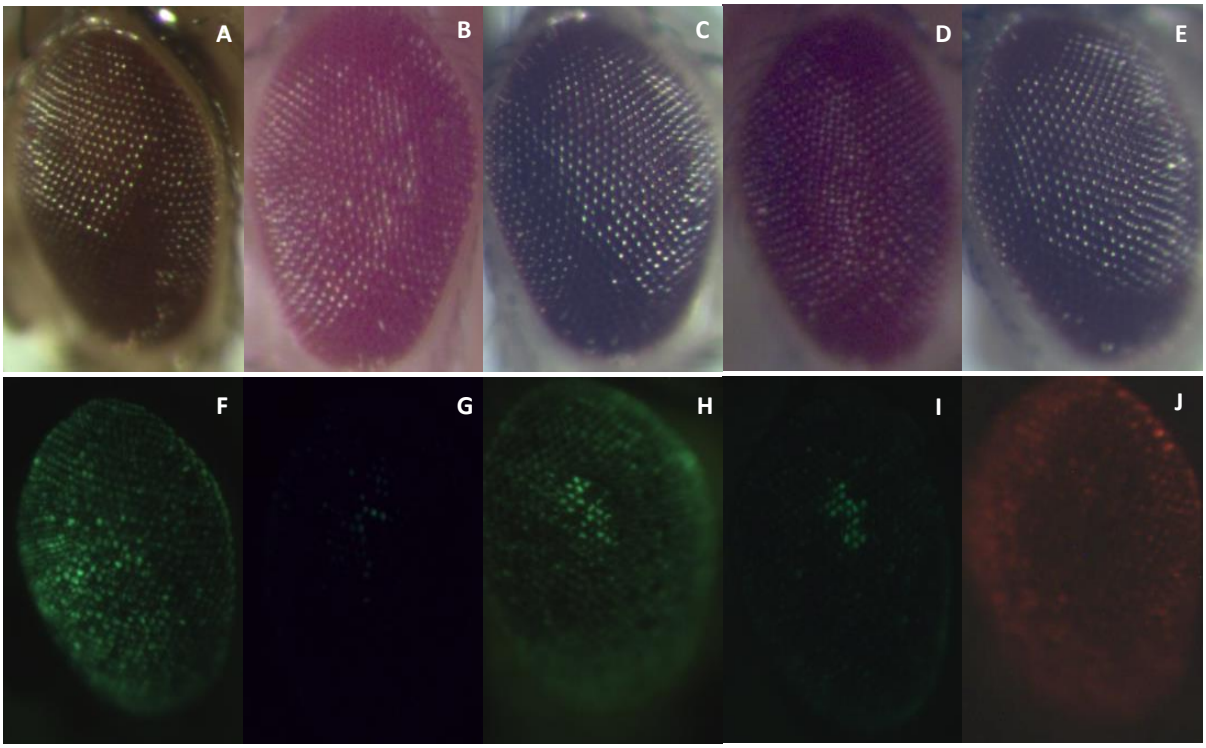


Figure 8. Ectopic expression of hSOD1-eGFP and hSOD1-mCherry in the adult eye under the eye-specific GMR-GAL4 driver.

Age: 30 days as adults. Note that all the ommatidia appear well-organized and healthy, with no obvious presence of aggregated hSOD1.

(A-E) are bright field images of the adult eyes that correspond to (F-J) of the same eyes under fluorescent illumination, respectively.

(A and F) wild type hSOD1-eGFP images.

(B and G) hSOD1-A4V-eGFP.

(C and H) hSOD1-G85R-eGFP.

(D and I) hSOD1-G93A-eGFP.

(E and J) hSOD1-G85R-mCherry.

Figure 9. hSOD1 expression under eye-specific GMR-GAL4

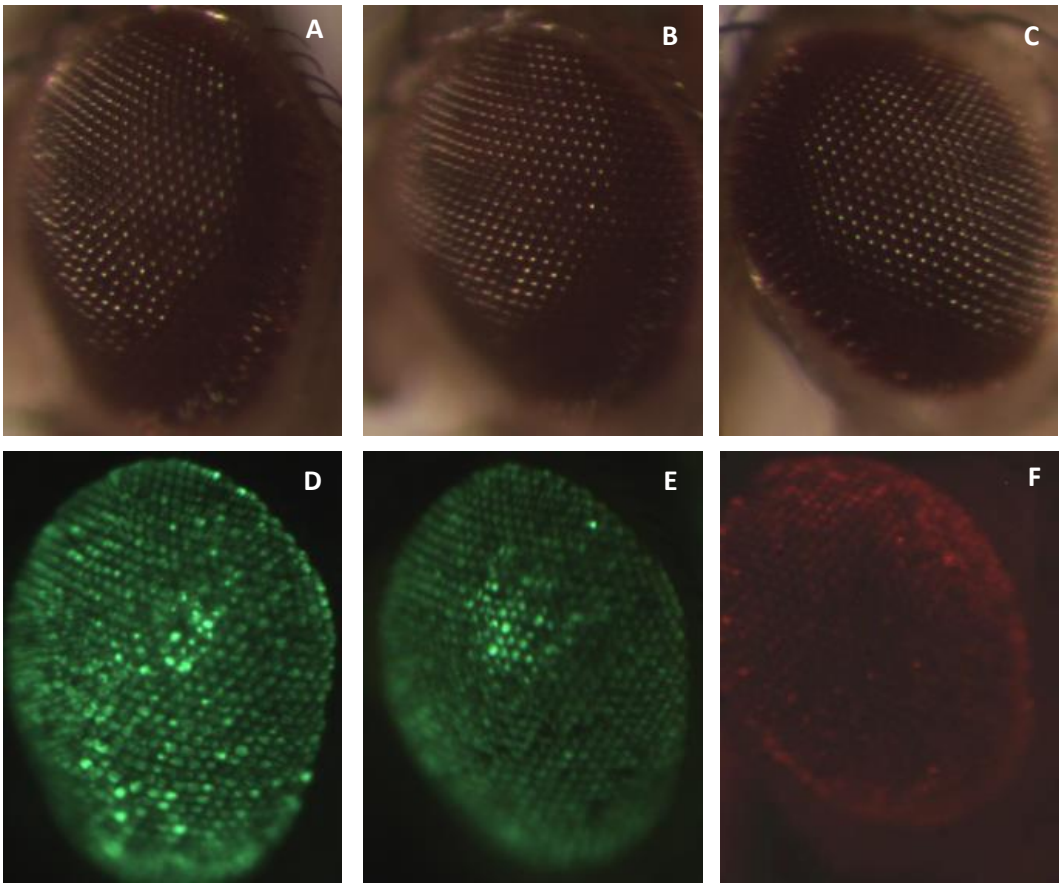


Figure 9. Ectopic expression of hSOD1-eGFP and hSOD1-mCherry in the adult eye under the eye-specific GMR-GAL4 driver.

Age: 70 days as adults. Note that the ommatidia appear well-organized and healthy, and there was no clear presence of aggregated hSOD1 in all genotypes.

(A-C) are bright field images of adult eyes that correspond to (D-F) the same eyes under fluorescent illumination.

(A and D) hSOD1-WT-eGFP.

(B and E) hSOD1-G85R-eGFP.

(C and F) hSOD1-G85R-mCherry.

When these hSOD1-expressing flies were visualized under fluoresecnt illumination to examine the expression of eGFP- or mCherry-tagged hSOD1, there was clear presence of the targeted proteins in the eyes. This observation is consistent with the confirmaiton by Western blot analyses, although their expression levels varied (Figures 3-5). For example, the level of A4V-hSOD1 expression was much lower as compared to that of other hSOD1 lines (Figure 3). It is possible that such variation is potentially due to positional effect of different inserational sites, or it could suggest a reduced stablibility of the A4V mutant hSOD1 protein. Further studies are needed to clarify this potentially interesting issue.

These exmainations also suggested that there were almost no visible aggregate formation in any of the hSOD1-expressing eyes (Figure 8-10), even in older flies at day 70 (Figure 4). Such an observation suggests that both wild type and either of the human SOD1 mutants do not easily form aggregates in *Drosophila* eye cells, which is in sharp contrast to the prominent aggregates formation by mutant Hutintingtin protein expressed under similar condition (Zhang et al., 2010),

To evaluate if co-expression of wild type hSOD1 could enhance the aggregation of mutant hSOD1 in *Drosophila,* as has been shown in mouse models (Deng et al., 2006), we simultaneously expressed wild type hSOD1-eGFP and mutant G85R-hSOD1-mCherry in the eye (**Figure 10)**. There was no observable enhancement of aggregation, although additional quantifition by Western blot remained needed to confirm this conclusion.

Figure 10. Co-expression of WT & G85R hSOD1 in the Eye

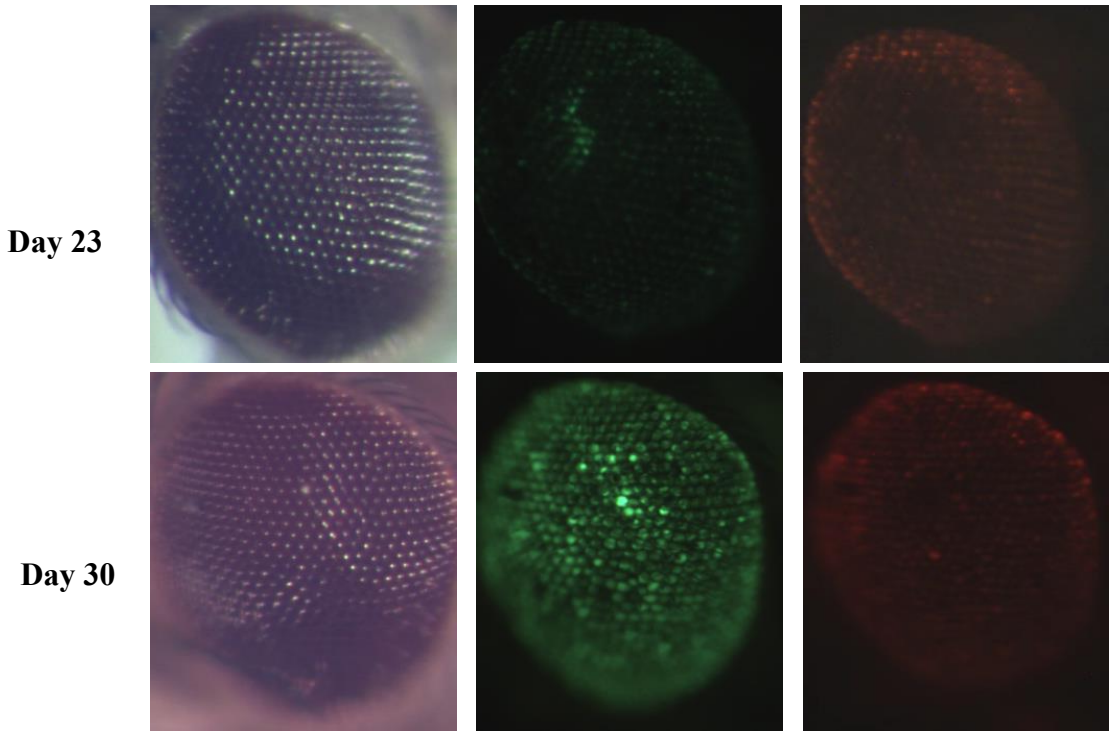


Figure 10. Co-expressing of wild type hSOD1 and mutant G85R-hSOD1 under eye-specific GMR-GAL4 driver.
Age: day 23 (top panels) and day 30 (bottom panels) as adults, as indicated.
From the left to right: the same adult eyes illuminated under bright field light (left, to reveal overall eye morphology), green fluorescent UV light (middle, to reveal wild type hSOD1-eGFP) and red fluorescent UV light (right, to reveal hSOD1-G85R-mCherry).
Note that the eye morphology appeared normal and there was no prominent aggregates in both eyes, although there appeared to be stronger SOD1-eGFP signals in the 30-day-old eye.

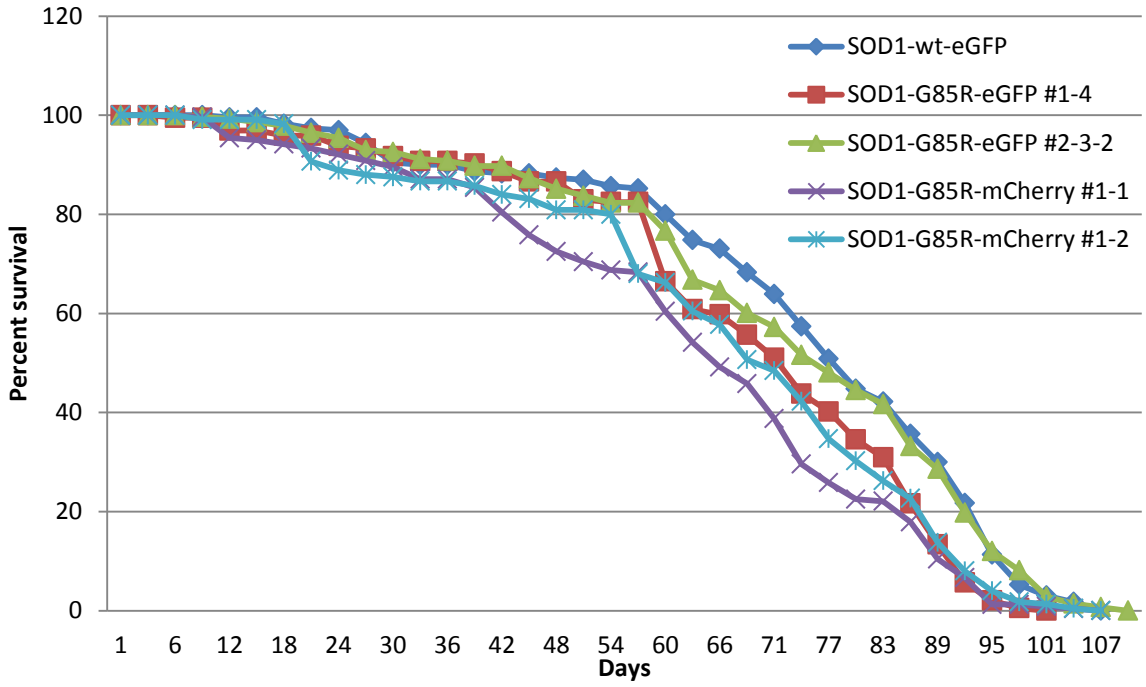
Motor neuron-specific expression of hSOD1

Considering that ALS is a motor neuron degenerative disease, we further selected the motor neuron-specific GAL 4 driver D42 to target hSOD1 transgene expression. This D42 driver will direct transgenic hSOD1 primarily in the motor neurons (Sanyal, 2009). Several assays can be performed to investigate the effects of hSOD1 expression on animal behavior, viability as well as the subcellular distribution and potential aggregation of wild type and mutant hSOD1 proteins. For example, the animals can then be monitored for locomotion defects such as the presentation or onset of paralysis. These animals can also be dissected to obtain the adult brain and ventral nerve cord and examined under fluorescent compound microscopy to determine the cellular localization and potential presence and localization of aggregation of ectopically expressed hSOD1 proteins. Stained with phlloidian to visualize the cytoskeleton and DAPI for the nuclei, the tissue can then be imagined with fluorescent microscopy. The fluorescent tagged hSOD1 proteins in this expression system should label the motor neurons and show, in addition to the pattern of expression, the formation of aggregates in the motor neurons if they occur.

The flies expressing hSOD1 directed by the D42 motor neuron GAL4 driver were aged for more than 90 days to assess their effects on animal locomotion and viability. To do this, the D42>UAS-hSOD1 flies were transferred to new vials with fresh food every 3 days to prevent animal loss related to texture changes in the food, and the number of the survival flies were counted at that time. Close observations of flies expressing mutation G85R under the D42 motor neuron driver showed no clear signs of paralysis or early decrease in mobility compared to hSOD1 wild type expression directed by the same driver. These observations are supported by survivorship data collected on G85R mutant hSOD1 fused to eGFP and

mCherry with respect to wild type hSOD1 fused to eGFP both expressed in motor neurons of the flies. As presented in **Figure 11** a total of 1,172 flies were counted, the mutant G85R-hSOD1 expressing flies, driven by D42-GAL4, could live up to 104 days as adults, similar as that of the wild type w^{1118} control (data not shown). Further, the overall trends of survival curve between wild type SOD1 and mutant G85R-hSOD1expressing flies closely resembled each other (Figure 6). Similarly, gross observations of mutant A4V- and G93A-hSOD1 expressing flies with D42 motor neuron revealed life span and mobility similar as that in WT-SOD1 flies (data not shown). Together, they suggest that animal mobility and life span are not significantly affected by mutant hSOD1 expression in *Drosophila* motor neurons.

Figure 11. Survival curve of adult flies expressing hSOD1 in motor neurons



We next carried out preliminary examination to check whether there existed protein aggregates formed by mutant SOD1 in these animals. Due to time restraints, we were only able to analyze flies expressing A4V-hSOD1 (**Figures 12 and 13**) and G93A-hSOD1 mutants (data not shown). From these flies, adult brain and ventral nerve cord were dissected and co-stained with phalloidin to visualize the actin cytoskeleton and DAPI dye for the nuclei. The image in Figure 7 is of a dissected adult fly brain and ventral nerve cord viewed at 10x magnification under fluorescent UV light to reveal of the expression of eGFP-tagged A4V-hSOD1. The image revealed areas of higher level expression of mutant A4V-hSOD1-eGFP. The brighter green areas in the image are actually the soluble A4V-hSOD1-eGFP expressed in motor neurons, (which, due to a high fluorescent background, might be confused as aggregation in a ubiquitous soluble A4V-hSOD1-eGFP expression context). When examined under fluorescent compound microscopy, there was clear presence of eGFP-tagged A4V- and G93A-hSOD1 protein in motor neurons that was enriched in the cell body and also distributed along the whole axonal tract (Figure 7 and data not shown). However, there was no prominent aggregate formation by mutant A4V-SOD1 protein in these neurons, even in flies of 42 days of age (**Figure 12**).

Figure 12. Adult brain and ventral nerve cord with motor neuron-specific expression of hSOD1

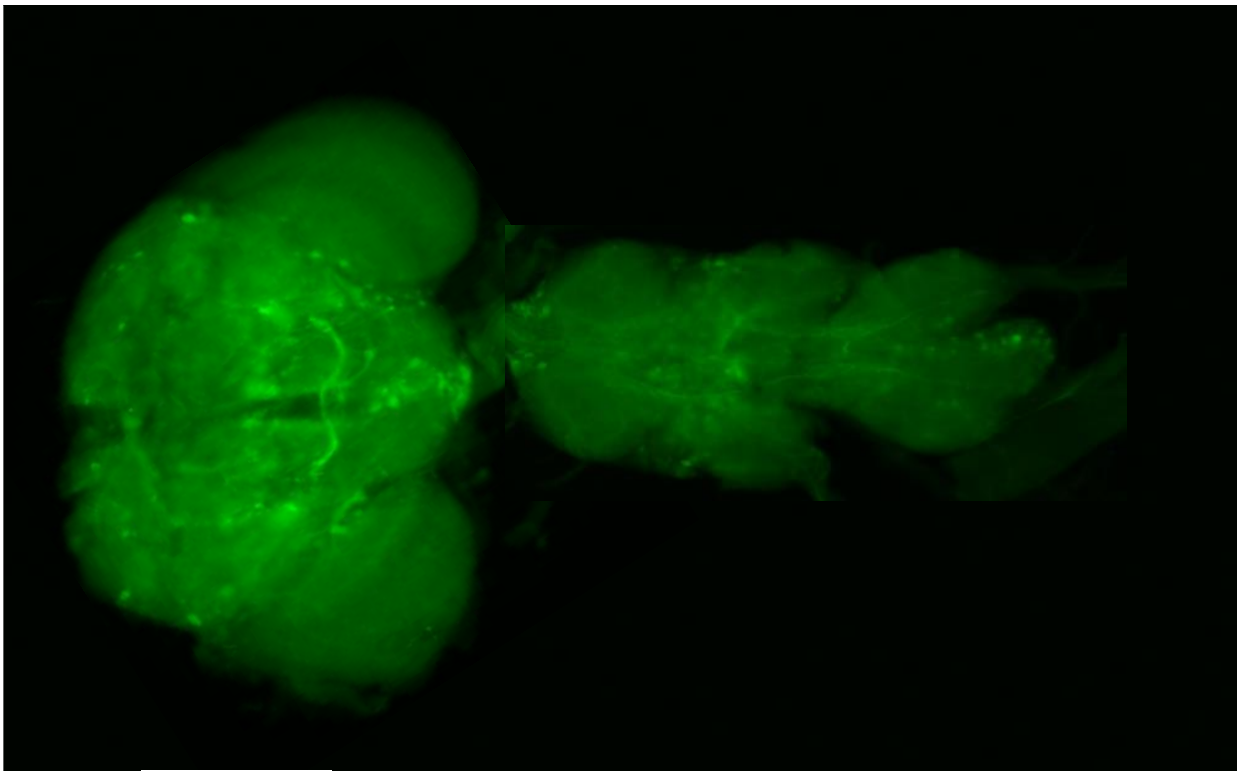


Figure 12. Fluorescent image of an adult fly brain and ventral nerve cord expressing mutant A4V-hSOD1 fused to eGFP, driven by the motor neuron-specific D42-GAL4.

In motor neurons, the expressed A4V-hSOD1-eGFP protein was enriched in cell bodies and also distributed evenly along the whole axonal tract. No prominent presence of aggregation of hSOD1 appears. Image was taken under10X magnification with Zeiss Axio Fluorescent compound microscopy.

Upon closer examination, we found a few structures resembling hSOD1-derived aggregates scattered along axon tracts of motor neurons (**Figure 13**). Some of these aggregates-like structures, enriched with eGFP-tagged mutant SOD1, also had accumulation of cytoskeleton protein F-Actin (Figure 8, top panels) while others did not (Figure 8, bottom panels). In the future, additional studies are needed to quantify these structures and determine whether such SOD1-enriched structures are indeed aggregates, for example by anti-ubiquitin antibody staining to see if they are modified by ubiquitination, a hallmark of aggregates from mis-folded proteins, or biochemical assay to examine their solubility in strong detergent such as SDS. However, from our studies both in motor neurons (Figures 7 and 8) and in the eye (Figures 3-5), it is clear that neither forms of the examined hSOD1 mutants develop robust aggregates in fly cells.

Figure 13. hSOD1 expression under the D42 motor neuron GAL4 driver

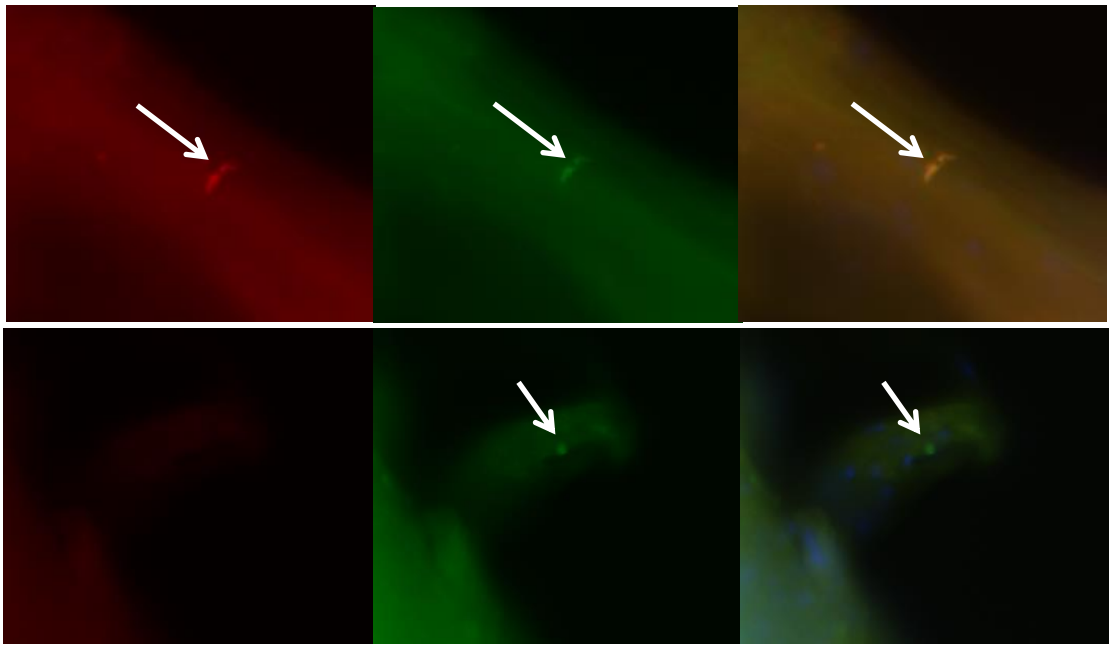


Figure 13. Aggregation of mutant hSOD1-A4V-eGFP in motor neuron axons

High magnification view of motor neuron axons with ectopic expression of hSOD1-A4V-eGFP (green) driven by motor neuron-specific D42 driver, which were co-stained with phalloidin (red) to reveal actin cytoskeleton (F-actin).

In top panels, SOD1-A4v-eGFP aggregates (green) were also enriched with F-actin (red), as revealed in overlaying image (right) and highlighted by white arrows .

Bottom panels are images of a motor neuron axon projecting from the ventral nerve cord. A SOD1-A4v-eGFP aggregate (green) was present but was not enriched with F-actin (red), as revealed in overlaying image (right) and highlighted by white arrow.

Image was taken under 40X magnification with Zeiss Axio Fluorescent compound microscopy.

Glia-specific over-expression of hSOD1 transgenes

ALS has been described a non-cell autonomus disease and as such neuron degeneration depends not only on mutant protein expression in the neuron but also in the surronding astrocyte/glia cells. Considering this, we next tested the effect of wild type and mutant hSOD1 expression in *Drosophila* glia cells. To do this, we crossed the established UAS-hSOD1 transgenic lines with glia cell-specific Gal4 driver line *gcm*-Gal4 (specific promoter from the *glia cell missing* gene), which is expressed in almost all glial cells including cortex, as soon as they are born. Cortex glia cells in *Drosophila* are also referred to as cell-body-associated glia and are very similar to vertabrate astrocytes (Freeman & Doherty, 2006). For this cross, a total of five line were selected for transgenic hSOD1 directed expression with the GCM GAL 4 driver. These line were two different G85R mutant lines fused to eGFP along with two lines of G85R fused to mCherry and one line of WT-hSOD1 fused to eGFP. Our observation were that very few, if any, flies expressing hSOD1 in under the GCM GAL4 driver, either mutant G85R or wild type survived to adult hood. Such a very interesting observation still needs furhter confirmation by using more independent hSOD1 lines, both for wild type and for different hSOD1 mutants. Also further testing should include additional glia-specific Gal4 driver such as *repo*-Gal4 as well as controls such as by pan-neuronal GAL4 driver *elav*-Gal4.

However, it does raise an intriging possibility that high level of hSOD1 expresssion in glia cells is lethal to *Drosophila*.

Summary and Discussion

We generated multiple UAS-hSOD1 transgenic lines and used available tissue-specific Gal4 lines, to carry out preliminary analyses of the toxicity and aggregation property of the wild type and mutant human SOD1 proteins in different fly tissue and cell types. Although the study is still not completed yet and more controls and assasys are needed to validate our preliminary observations. It is apparent at this point that human SOD1 protein, either as wild type or the three mutated forms we examined, does not form robust aggregates in different test settings. Included in our study is expression in cultured *Drosophila* S2 cells, in adult eyes over an extended peroid of time (up to 70 days) or in motor neurons.

The original purpose of this project is to assess *Drosophila* as a model system to study aggregates by mutant hSOD1, and if successful, its long term goal is to identify regulators of aggregate formation by mutant hSOD1. To faciliate these goals, our expression paradigm incorperates the fusion of a green or red fluorsecent protein to SOD1 proteins to allow easy visualization of SOD1 protein expression and their aggregation. However, based on our preliminary results so far, hSOD1 does not easily form aggregates in either the S2 cell model or whole animal model in *Drosophila.* We will need to take serious re-consideration regarding the viability of pursueing hSOD1 mutants in this model system.

Nevertheless, results so far also raised several interesting questions. For example, although both mutant Huntingtin and mutant SOD1 proteins form aggregates in human cells, they clearly behave very differently in term of aggrgates formation in *Drosophila* system.

Such a different behavior suggests that protein properties, and potentially aggregating dynamics, of mutant Huntingtin and mutant SOD1 are very different. Moreover, why even mutant forms of human SOD1 protein do not form robust aggregates even when expressed at a high level over an extended period of time? One possibility is that protein quality control machineries in *Drosophila* cells, those involved in protein re-folding and in clearance of mis-folded proteins, are more effective than their counterparts in mammalian systems in dealing with mutant SOD1 proteins. If this hypothesis is true, it raises a possibility that we can identify the machinery(ies) in *Drosophila* that can correct or clear the mis-floded mutant human SOD1 proteins, and harness its working mechanism to treat ALS.

Another very intriguing observation is the lethality caused by the overexpression of either wild type or mutant G85R-hSOD1 specifically in glia. Notably, overexpression of these proteins in the eye or motor neurons do not lead any obvious detrimental effect. Although furhter studies are required to confirm the above observations, they raise a very intersting possibility that glia cells are especially vulnearble to SOD1 expresion. Moreover, existing findings from mammalian studies already support that nonautonomous effect from astrocyte and glia contribute significantly to the ALS pathogenesis. Together, they present a question whether the lethality associaited with glia-specific expresssion of SOD1 share some mechanistic similarity with the pathogensis underlying ALS.

CHAPTER SEVEN

Perspectives

Toxicity and aggregation of human SOD1 protein in *Drosophila*

The results obtained during the study of this thesis project were consistent with a previously established ALS models using hSOD1 in *Drosophila* (Watson et al., 2008). In that study wild type hSOD1 and hSOD1 with A4V and G85R mutations were examined for neuronal toxicity in *Drosophila*. Expression of human SOD1 in Drosophila was directed by the GMR (eye specific) and D42 (motor neuron) Gal 4 drivers. The flies were examined for deficits in motor function and life span, as well subject to Western analysis, motor neurons counts and electrophysiology. It was found that although progressive climbing difficulty was recorded with both wild type and mutant hSOD1 expression in motor neurons, there was no significant loss of motor neurons nor obvious signs of paralysis (Watson et al., 2008). Toxicity of wild type hSOD1 was confirmed with electrophysiology studies and showed similar defect to that seen in G85R flies. The results suggest the giant fiber circuit is defective when given high frequency stimulation, revealing defects in synaptic transmission of older flies expressing wild type and G85R mutant hSOD1 in motor neurons (Watson et al., 2008). Further, their life span data for motor neuron directed expression of WT and mutant hSOD1 flies revealed similar survivorship curves as their control flies which expressed GFP only under the same D42 driver (Watson et al., 2008). Finally, they found that focal accumulation of hSOD1 protein did occur when directed by the eye-specific GMR-Gal4 and motor neuron-specific D42 drivers, and that in motor neurons it was accompanied by a stress response in surrounding glia cells (Watson et al., 2008). Interestingly, the study did observe the same change to migration rate of mutation G85R of hSOD1 in western analysis (Watson et al., 2008). Interestingly, the study did observe the same change to migration rate of G85R-hSOD1 mutation in Western analysis (Watson et al., 2008). Notably, they concluded that

neuronal toxicity was not the result of hSOD1 aggregates and hSOD1 expression did not cause a loss of neurons (Watson et al., 2008). Our current study, with respect to a lack of obvious paralysis and unaffected life span in mutant hSOD1-expressing flies as compared with ones with wild type hSOD1 expression, is consistent with this previous work.

The previously established model by Watson *et al*, however, did not employ hSOD1 fused to eGFP or mCherry fluorescent reporters, in contrast to our model. Thus, this suggests that the fusion of a fluorescent protein to wild type and mutant hSOD1 does not increase or decrease its toxicity in *Drosophila*. In addition, fusing fluorescent proteins to hSOD1 does not appear to adversely affect the solubility and subcellular localization of hSOD1 in *Drosophila*. Although the Waston *et al* 2008 study reported the toxicity of SOD1 protein in the fly, as manifested as a decrease in geotaxis response (Watson et al., 2008), we have yet to confirm this observation using our SOD1 model. However, we did observe the presence of aggregates-like structures in neurons of SOD1-expressing animals when examined under fluorescent microscopy (Figure 7). Further studies are needed to clarify this observation.

Glia-specific toxicity of human SOD1 protein in *Drosophila?*

Interestingly, in our current study, when wild type or mutant hSOD1 transgenic flies were crossed with a glia cell specific GAL4 driver, *gcm*-GAL4, a surprising lethality phenotype emerged that suggests expression of hSOD1 in this tissue could be lethal. ALS caused by SOD1 mutations in general has been described as a non-cell autonomous disease (Boillee, Vande Velde, et al., 2006; Boillee, Yamanaka, et al., 2006; Da Cruz & Cleveland, 2011; Nagai et al., 2007; Tamura, Sone, Yamashita, Wanker, & Okazawa, 2009; Wallis et al., 2012). Non-cell autonomy is a term here to describe the nature of toxicity as dependent

on the involvement of surrounding cell or tissues. Glia and astrocyte cells are known as support cells for neurons but recently their true nature of involvement with functions of neurons and synaptic transmission has been revealed to be very complex (Volterra & Meldolesi, 2005). It is now known that astrocytes are indeed excitable and express in their membrane many of the same receptors and channels expressed by neurons (Volterra & Meldolesi, 2005). Perhaps most notably astrocytes play a major role in sequestering glutamate from the synaptic cleft, to avoid it remaining in the cleft and causing excytotoxicity of neurons. The loss of the astrocyte glutamate reuptake transporter EAAT2 has been seen in ALS and is believed to be a major contributor of glutamate related excytotoxicity (Howland et al., 2002; Wallis et al., 2012). In addition astrocytes release trophic factors to neurons such as TNFα, which induces insertion of AMPA receptors into the plasma membrane (Yin et al., 2012). AMPA receptors are the key metabotropic excitatory receptors for which the agonist is glutamate. However, in advanced stages of the disease progression the increased in TNFα release by astrocytes becomes toxic to neurons (Yin et al., 2012).

Future studies based on the established SOD1 models in *Drosophila*

More work can be carried out to further test the above hypotheses using our hSOD1 models. First, in the whole animal models, it should include ubiquitous expression of both wild type and mutant hSOD1, to more comprehensively determine the presence of an aggregation phenotype. In doing so, we can also determine whether the hSOD1-induced lethality phenotype observed under the glia cell *gcm*-GAL4 driver is truly glia-specific.

Second, of the current SOD1 model in S2 cells, which does not form robust aggregates, it is possible that *Drosophila* S2 cells are expressing a gene alone or of a pathway that is capable of completely suppressing aggregate formation by mutant hSOD1. In such a scenario, our S2 cell-based SOD1 models could be used to conduct the proposed genome-wide RNAi screen to reveal such a *protective* gene or pathway(s), potentially leading to a new and exciting research direction.

Further, *Drosophila* S2 cells are macrophage lineage cells, which are immune system cells, not neuronal cells. Since ALS is a neurodegenerative disease, switching to a *Drosophila* neuronal cell line may yield different results with regard to aggregate formation. With regard to copper homeostasis, *Drosophila* S2 cells contain copper chaperones that are powerful regulators of free copper (Southon, Burke, Norgate, Batterham, & Camakaris, 2004). hSOD1 has been shown to accumulate and aggregate in neurons, astrocytes and mitochondria (Cozzolino et al., 2009; Jaiswal et al., 2009; Nguyen et al., 2009). Aggregation of any protein in a cell is a concentration dependent process, which means it inherently requires accumulation of significant amount of mis-folded proteins and thus the expression level of the protein will be an important factor in aggregation process. On this aspect, our S2 cell-based model employs copper induction of hSOD1 expression, while an enzymatically active hSOD1 by itself is required to complex with copper and zinc, thus titration of aqueous copper may be necessary to optimize its hSOD1expression. Another possibility would be to codon optimize the inserted hSOD1 gene, to the codons preferred by the *Drosophila* species. This has been suggested by the advising committee over this project and is postulated to result in a noticeable difference in phenotype presentation.

Cellular toxicity in ALS: a link between dysregulated neuronal excitability, aggregates and others?

The underlying cellular and molecular mechanisms underlying ALS are still far from clear. Nevertheless, it is quite intriguing to observe a link between dysregulated neuronal excitability, aggregates formation and other cellular factors in ALS pathogenesis.

To facilitate normal neuronal communication neurons must generate action potentials to allow an influx of calcium, which then signals the release of neurotransmitter to the synaptic cleft. Action potentials are propagated by and result from the opening of voltage gated sodium channels, which allow for an influx of positively charged sodium ions, thereby depolarizing the neuron. This sodium current allows for a spike in voltage, which is propagated down the axon to the synaptic cleft. That action causes the necessary increase in calcium concentration, which then facilitates the binding of vesicles to the inner membrane of the neuron, subsequently the vesicle fuses with the membrane and opens to the synaptic cleft to release its neurotransmitter contents. The neurotransmitter molecules travels across the synaptic cleft from the presynaptic neuron to the post synaptic neuron and eventually cause either an inhibitory postsynaptic potential or and excitatory post synaptic potential (IPSP or an EPSP).

All neurons maintain a negatively polarized resting membrane potential that is caused by negatively charged proteins and DNA. The resting membrane potential is maintained at around -65mV by high intracellular potassium concentration. However, for an action potential to occur the membrane must depolarize to a certain threshold potential and then an all or nothing response in the form of an action potential is generated. An increase in intracellular chloride, mediated by GABA channels, can prevent current from cation channels

depolarizing the membrane. This action will then inhibit the neuron from reaching the depolarized threshold necessary to generate an action potential. Increases in depolarizing threshold have been seen in ALS motor neuron axons and it has been suggested to be attributed to dysfunction in either expression of or conductance of potassium channels. (Cheah et al., 2012; Shibuya et al., 2011) However, an accumulation of negatively charged proteins could be the underlying cause of that dysfunction and subsequent increase in excitability. hSOD1 has a net negative charge of -6 and mutated SOD1 maintains the same charge or in some cases decreases, but only slightly, while in other cases such as G127X the net negative charge increases. (Sandelin, Nordlund, Andersen, Marklund, & Oliveberg, 2007) Mutated proteins, such as SOD1, at some point lose functionality and accumulate while remaining soluble and then aggregate. In this quasi-stable state of soluble accumulation the negatively charged proteins may be able to aberrantly contribute to the global negative charge of a cell. This may result in changes to cation conductance and produce excessive inhibition of action potentials. Thus, the neuron in cooperation with astrocytes struggles to overcome excessive inhibition and in the process becomes over excitable.

In support of the above hypothesis, in addition to the dysfunction seen of potassium channel in ALS models, the following findings are noteworthy. First, it has been shown that some mutant forms of SOD1 develop less soluble yet toxic aggregates, then become more toxic when soluble yet mis-folded, but then becomes less toxic when correctly folded by chaperones.(Brotherton, Li, & Glass, 2012). The less soluble but more aggregated SOD1 mutants become very toxic when solubilized until correctly folded by chaperones (Brotherton et al., 2012). This evidence supports that mutant SOD1 is most toxic when mis-folded and accumulated while still in electrochemical contact within the cell. Second, an increase in

persistent inward sodium conductance has been seen in ALS and causes increased excitability. (Kiernan, 2009; Meehan et al., 2010; Shibuya et al., 2011) Third, TNFα release by astrocytes is increased which actually induces AMPA receptor insertion into the membrane, while later with disease progression it becomes toxic. (Boillee, Vande Velde, et al., 2006; Yin et al., 2012) Overexpression of AMPA receptors will markedly increase excitability and has been well established to play a prominent role in ALS. Fourth, astrocytes have been shown to release d-serine which is a stronger agonist for NMDA receptors than glycine and can actually increase their ability to conduct cations. (Sasabe et al., 2007) NMDA up regulation in this manner has been seen in ALS and leads to an increase in excitability, which in turn leads to an increased cytoplasmic calcium concentration (Sasabe et al., 2007; Spalloni, Nutini, & Longone, 2013) Fifth, the loss of EAAT2 glutamate transporter in astrocytes results in an increase in the concentration of the main excitatory neurotransmitter glutamate in the synaptic cleft, which in turn will cause excessive activation of NMDA and AMPA receptors (Howland et al., 2002). Consequently, again leading to increased calcium concentration in the neuron, a sign of excytotoxicity, and a result of increased excitability.

ALS and athleticism: a causative or an incidental link?

Some studies have shown a correlation between prolonged strenuous activity, such as athletics or occupations that require intense physical activity and onset of ALS (Scarmeas, Shih, Stern, Ottman, & Rowland, 2002; Sutedja et al., 2009). One most famous case of ALS is Lou Gehrig, an outstanding baseball player who fell victim to the ALS during the peak of his career, and ALS has often or better known by general public, as Lou Gehrig's disease. Notably, in ALS, the changes to neurons with regard to sodium and potassium channel

conductance are seen well before symptoms start and are suggested to appear early in life. If there is a higher degree of excitability in motor neurons that occurs well before onset of symptoms, it could translate to an increase in stamina. High stamina can lead to success when engaged in physically demanding activities such as athletics. Just as a general increase in energy level that may be the underlying reason for a lower body mass index (O'Reilly et al., 2013). There is a remote possibility that it may not be exercise or environmental factors that contribute to ALS; it may be that the underlying cellular mechanisms of ALS make patients more likely to be active at a younger age.

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Vita

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