Caloric restriction and SirT1 modulate APP metabolism *in vitro* and *in vivo*

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Dedications

I would like to dedicate this dissertation to my husband, Anshuman Chakraborty, for his tremendous support and encouragement, my daughter Anushka, my parents, who always taught me to dream big and believed in me, and my in-laws for their love and support.

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Abstract

Proteolytic processing of the β-amyloid precursor protein (APP) by α-, β-and γ-secretase enzymes generating the amyloid-beta (Aβ) peptide and the APP intracellular domain (AICD) is a central event in Alzheimer's disease (AD). Herewe show that *in vitro* CR decreases Aβ, AICD and full-length APP levels in human cell lines without affecting APP transcription and that some of these effects can be recapitulated by over-expressing the NAD^+ dependent deacetylase SirT1 in our cell lines. Resveratrol, a SirT1 agonist, also has similar effects on APP metabolism. SirT1 and resveratrol however, do not affect full-length APP levels. In our cell lines, SirT1and resveratrol reduces secreted Aβ levels by increasing the α-secretase cleavage of APP and also possibly by affecting γ-secretase activity. Extending these studies to an *in vivo* setting, an AICD reporter *Drosophila* model of AD, shows that caloric restriction, Sir2 gain-of-function and resveratrol treatment suppress AD-like rough-eye phenotype in the fly eyes. Finally to study the mechanism of CR and SirT1 mediated effects on APP metabolism in entire central nervous system, we created and characterized a novel *Drosophila* model of AD. We have shown that our model displays neuroanatomical and behavioral features that are characteristic of AD patients.

CHAPTER 1: INTRODUCTION

Background and Significance:

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. It is considered to be the most common cause of dementia, especially in the elderly population. It affects a person's memory, mood and behavior. It seriously affects a person's ability to carry out daily activities (LaFerla and Oddo 2005). It involves parts of the brain that control thought, memory and language. Since age is the biggest risk factor for AD, Frequency of AD has increased over the past 40 years because people are living long enough to develop the disease (Evans, Smith et al. 1991). Taking the increases in life expectancy into consideration it has been predicted that by the mid century, the number of people living with AD will quadruple (Yaari and Corey-Bloom 2007). To date there is no cure for the disease. Current therapies only treat the symptoms and not the underlying causes of the disease. It is very important to understand the molecular events leading to the development of AD in order to predict who will develop AD and develop potential therapeutics.

AD Pathology

The pathological features of AD include the presence of senile plaques, neurofibrillary tangles, along with a massive loss of neurons, primarily in the cerebral cortex and hippocampus. The senile or amyloid plaques are extracellular deposits composed of a small peptide (~4 kD) called β-amyloid (Aβ), surrounded by dystrophic neurites, reactive microglia and astrocytes(LaFerla and Oddo 2005).The neurofibrillary tangles are intraneuronal lesions consisting of 10nm thick paired-helical filaments. The

main component of these filaments is the hyperphosphorylated form of the microtubule-binding protein Tau(Grundke-Iqbal, Iqbal et al. 1986; Goedert, Wischik et al. 1988).

The amyloid-cascade hypothesis is the leading hypothesis for the cause of AD. This hypothesis proposes that Aβ accumulation is the initiating event for development of AD and that the tau pathology and other degenerative changes are a downstream consequence of the Aβ accumulation (Hardy and Selkoe 2002). Proposed approximately twenty years ago, considerable experimental evidence has confirmed a central role of Aβ in initiating the AD pathogenic cascade and argues that the neurodegenerative disease process, including the development of neurofibrillary tangles, is a consequence of imbalance between the generation and clearance of Aβ. The Aβ peptides are generated by proteolytic processing from a larger β-amyloid precursor protein (APP) (De Strooper and Annaert 2000). *APP* gene maps to the long arm of human chromosome 21 and codes for a type 1 integral membrane protein. APP can be proteolytically processed in different ways by specific secretase enzymes producing different fragments including the Aβ peptides of the amyloid plaques.

Risk Factors of AD

There are several risk factors for developing AD. Age, genetics and life history are some of the important risk factors for developing AD, of which, age is by far the most important. Ninety percent of AD cases are deemed late-onset AD (LOAD), when people develop the disease after 65 years of age.

In addition to this age component, LOAD also has a genetic component. Polymorphisms in the *ApoE* gene have been consistently linked to LOAD. The ε4 allele of Apolipoprotein E is the major risk factor for a majority of late-onset AD cases (Bu

2009). In humans there are three different allelic forms of the *APOE* gene: ε2, ε3 and ε4. The ε4 allele frequency is 15% in general populations but is 40% in AD patients. Individuals with one ε4 allele are three to four times more prone to develop AD, than those without ε4 alleles {reviewed in (Bu 2009)}. Risk for developing AD increased four-fold and mean age at onset decreased from 84 to 68 years with twoε4 alleles in families with late onset AD. Thus APOE-ε4 gene dose is a major risk factor for LOAD (Corder, Saunders et al. 1993).

Early onset Alzheimer's disease (when people develop the disease before 65 years of age) depends on family history. Three genes are associated with early onset form of AD: *APP* (chromosome 21), *Presenilin-1*(chromosome 14) (PS-1), and *Presinilin-2* (PS-2) (chromosome 1). Mutations in these genes are inherited in an autosomal dominant manner. Individuals who carry these mutations are predisposed to develop AD at a relatively early age. Although these mutations account for ~5% of all AD cases, they all affect APP processing and Aβ production. Mutations in the APP gene on chromosome 21 either increase total Aβ levels or just $A\beta_{42}$ (the more neurotoxic form of the Aβ peptide) alone, and some mutations in the central part of the Aβ sequence lead to the formation of more dense aggrerates (Tanzi and Bertram 2005; Findeis 2007; Bertram and Tanzi 2008). Again, in Down's syndrome (DS, trisomy 21), an extra copy of chromosome 21 and of the *APP* gene results in increased APP expression as well as increase in Aβ levels, which lead to AD-like deposition of amyloid plaques in those DS patients (Findeis 2007).Most familial mutations in *APP, Presenilin 1 and Presenilin 2* genes lead to over-production of $A\beta_{42}$, a larger and more neurotoxic form of Aβ. There are several other risk factors for developing AD.

Many studies have shown that vascular risk factors including diabetes, hypertension, dyslipidemia, and obesity are risk factors for developing dementia. In addition, people with depression have been shown to be at high risk for cognitive impairment. Population studies have reported that intake of antioxidants or polyunsaturated fatty acids reduce the incidence of dementia, and it has been reported that people who are cognitively, socially, and physically active have a reduced risk of cognitive impairment (Middleton and Yaffe 2009). Among other non-genetic factors influencing AD, recent studies strongly support the evidence that caloric intake may play a role in the relative risk for AD clinical dementia. Indeed, the effect of diet on AD pathogenesis has been an area of research that has produced promising results. Studies in different AD models lend support to the hypothesis that diet is a major risk factor and caloric restriction might help prevent the development of late onset AD. In fact it has been shown by several groups that caloric restriction decreases Aβ production (Wang, Ho et al. 2005; Qin, Chachich et al. 2006; Halagappa, Guo et al. 2007), which is produced by proteolytic processing of APP.

Amyloid Precursor Protein

Mammalian APP has three different isoforms: APP, and APP Like Proteins APLP1 and APLP2 (Sprecher, Grant et al. 1993). *APP* gene is located on Human Chromosome 21 and codes for a type 1 integral membrane protein which has a large extracellular domain and a shorter intracellular domain and 7 membrane spanning transmembrane segments. APP is highly expressed in brain and in most other tissues and it belongs to a family of proteins found in vertebrates, *Drosophila*, and *C. elegans.* The mammalian APP proteins are relatively ubiquitously expressed. The *APP* gene contains

19 exons, of which, exons 7, 8 and 15 can be alternatively spliced (De Strooper and Annaert, 2000) to produce different isoforms of the protein of which APP695, APP $_{750}$ and APP$_{771}$ are the most predominant. APP $_{750}$ and APP$_{771}$ contain an additional56 aa domain that is structurally and functionally related to Kunitz-type serine proteinase inhibitors (KPI)(Ponte, Gonzalez-DeWhitt et al. 1988). APP$_{695}$ (containing 695 aminoacid residues) is the most dominant form in neurons; whereas the other two isoforms are more prevalent in non-neuronal cells, especially in glial cells. APP is proteolytically processed in different ways to produce different fragments including the Aβ (Amyloid-beta) peptides of the amyloid plaques. Depending on whether Aβ peptide is producedor not, the proteolytic processing of APP is directed towards the non-amyloidogenic pathway or amyloidogenic pathway.

*APLP1*and *APLP2* genes map to human chromosome 19 and chromosome 11 respectively. Both APLP1 and APLP2 are also membrane-associated glycoproteins that can undergo Regulated-Intramembrane-Proteolysis (RIP) by γ-secretase in a manner similar to APP cleavage (Gu, Misonou et al. 2001). It has been shown that PS1 deficiency led to elevated levels of APLP1 C-terminal fragments in brains of animal models (Naruse, Thinakaran et al. 1998). Both APLP1 and APLP2 can be proteolytically cleaved by α- and γ-secretase producing the respective C-terminal fragments with can further undergo cleavage at the ε-site releasing the intracellular fragments (comparable to AICD). Studies in SH-SY5Y cells have shown that along with the intracellular fragment γ-secretase cleavage of APLP1 produces a p3 like fragment, where as APLP2 can produce an Aβ-like fragment and a p3-like fragment (Eggert, Paliga et al. 2004). Animal studies have shown that mice with single knock-out of *APP* (Zheng, Jiang et al. 1995),

APLP-1 (Heber, Herms et al. 2000), or *APLP-2* (von Koch, Zheng et al. 1997) gene do not display any severe phenotype, suggesting that the mammalian *APP* gene family members have a redundant function. However, APLP-1(–/–)/APLP-2(–/–) and APP(–/–)/APLP-2 (–/–) double knock-outs are lethal, but mice with APP(–/–)/APLP-1(–/–) double knock-outs are viable(Heber, Herms et al. 2000), suggesting that APLP-2 plays an important role during embryonic development.

Not much is known about the physiological functions of APP. Some studies have shown that the secreted fragments of APP that contain the KPI like domain are similar to the cell-secreted proteinase inhibitor known as protease nexin-2(PN2) (Van Nostrand, Wagner et al. 1989).APP can also inhibit some serine proteinases, like the prothrombotic enzymes factor XIa, factor IXa, factor Xa, and tissue factor: factor VIIa complex(Smith, Higuchi et al. 1990; Van Nostrand, Wagner et al. 1990; Schmaier, Dahl et al. 1993; Mahdi, Van Nostrand et al. 1995).These studies suggest that APP plays a regulatory role in the blood coagulation pathway. Recent studies have shown that over-expression of PN2/APP either in platelets or in brains of transgenic mice lead to inhibition of thrombosis and development of larger hematomas in experimental intracerebral hemorrhage suggesting APP plays a significant role in regulating cerebral thrombosis (Xu, Davis et al. 2005). Functions of other proteolytic fragments of APP have been described later.

As mentioned earlier, APP (all the different isoforms) undergoes *N*- and *O*-glycosylation within its extracellular/luminal domain. The N-glycosylated form of APP is localized in the endoplasmic reticulum (ER) and golgi and is referred to as the immature APP (ImAPP) and is not subjected to proteolysis by the secretases (Tomita, Kirino et al.

1998). The ImAPP is further transported through the golgi where it undergoes O-glycosylation. This N- and O-glycsylated form is referred to as mature APP (mAPP). From the trans-Glogi network, mature and immature APP enter the late secretory pathway **(Figure 1-1),** where mAPP is subjected to cleavage by α– and/or β-secretases on the extracellular/lumenal domain and γ-secretase in the transmembrane domain (Small and Gandy 2006). It has been shown that APP_{695} is the main form of APP detected from whole brains indicating that Aβ is mainly generated from neuronal cells. Active β-secretases are highly concentrated in the late secretory pathway, especially in the endosomal-lysosomal pathway, where Aβ is generated from mAPP **(Figure 1-1)**. Thus Aβ generation is closely related to APP trafficking in the cell, especially in neurons(Small and Gandy 2006). The C-terminal fragment of APP contains phosphorylation sites and functional motifs that play an important role in the regulation of its metabolism, trafficking, and function. Cleavage of mAPP by α- and β-secretases can potentially produce three different C-terminal fragments (C99, C89, and C83; numbers indicate amino acid number) in the brain.Both C99 and C89 (also referred to as CTFβ and CTFβ)are products of cleavage by β -secretase cleavage of APP and can be producedbased on the abundance of the enzyme whereas α-secretase cleavage produces a slightly bigger fragment (C83 or CTFα)(Liu, Doms et al. 2002). Some APP CTFs are phosphorylated at Thr^{668} and detected as phosphopeptides pC99, pC89, and pC83(Buxbaum, Thinakaran et al. 1998). The phosphorylation state of APP seems to be important for its trafficking functions.

APP is also suggested to be a part of several physiological processes including in cell proliferation, cell survival, neuroprotection, enhancement of memory, neuronal

excitability and regulation of synaptic plasticity (Ma, Bagnard et al. 2008). It has been further shown that over-expression of sAPPα (the secreted fragment of APP released from α-scretase cleavage) was sufficient to recover anatomical, behavioral and electrophysiological abnormalities of APP-deficient mice (Ring, Weyer et al. 2007) suggesting that sAPPα has a neuroprotective function. sAPPα also controls neural stem cell division in the adult subventricular zone (Conti and Cattaneo 2005). The secreted fragments of APP have also been reported to stimulate neural stem cell proliferation (Hayashi, Kashiwagi et al. 1994).

Regulated Intramembrane Proteolysis of APP

Regulated Intramembrane Proteolysis (RIP) involves several proteases that cleave different regions of the membrane bound APP protein. The initial cleavage is referred to as ectodomain shedding and occurs within the ectodomain at a peptide bond close to the transmembrane domain (TMD). Shedding releases the ectodomain outside the cell and generates a membrane-bound stub, which then undergoes a second cleavage within its TMD, called intramembrane proteolysis. Members of the disintegrin and metalloprotease (ADAM) family, matrix metalloproteases and the aspartyl proteases β-site APP-cleaving enzymes 1 and 2 (BACE1 and BACE2) carry out the ectodomain-shedding step (Lichtenthaler and Steiner 2007). Depending on whether Aβ peptide is produced or not, the proteolytic processing of APP is directed towards the non-amyloidogenic pathway or amyloidogenic pathway. The principal ectodomain proteolytic cleavage of APP is performed by **α-secretase.** The α-secretase cleaves APP between Lys16 and Leu 17 (residues 612 and 613 of APP) of the Aβ sequence itself and therefore precludes the generation of Aβ peptide, the non-amyloidogenic pathway.α-secretase activity is a

carried out by a family of zinc metalloproteinases that include several members such as TACE/ADAM17, ADAM9, ADAM10 and MDC-9.

In amyloidogenic processing, APP is cleaved initially by **β-secretase.** The major neuronal β-secretase is a transmembrane aspartyl protease termed BACE1 (β-site APP-cleaving enzyme 1)(Vassar 2004). BACE1 cleaves at the N-terminus of the Aβ sequence generating a soluble fragment called sAPPβ that is released outside the cell and a C-terminal fragment called C99 or CTFβ that remains tethered to the membrane **(Figure 1-2).** BACE1 can also cleave within the Aβ domain between Tyr^{10} and Glu^{11} (β′-cleavage site) (Thinakaran and Koo 2008).The high neuronal expression of BACE1 preferentially channels APP through the amyloidogenic processing pathway in the brain.

The proteolytic products of the amyloidogenic and non-amyloidogenic processing pathways, CTFα and CTFβ fragments, are subsequently cleaved within the transmembrane domain by γ-secretase to release Aβ (4 kDa) and p3 (3 kDa) peptides, respectively. These fragments are also released outside of the cell. In addition to Aβ, γ-secretase cleavage generates a cytoplasmic polypeptide termed as APP Intra Cellular Domain (AICD) (Thinakaran and Koo 2008) **(Figure 1-2)**. γ-secretase activity is mediated by large protein complex composed of four different components: presenilin-1 or -2, nicastrin, APH-1, and PEN-2(Iwatsubo 2004). γ-Secretase cleaves CTFβ at multiple sites, generating Aβ peptides ranging in length from 38 to 43 residues(Selkoe and Wolfe 2007). Almost 90% of secreted Aβ is in the $Aβ_{40}$ form, whereas $Aβ_{42}$ accounts for <10% of secreted Aβ. The majority of familial AD-linked APP mutations are located C-terminal of the Aβ domain, close to the γ-secretase cleavage sites, increase $Aβ_{42}$to $Aβ_{40}$ ratio. Mutations in presenilin-1 and -2 also influence γ-secretase cleavage in

a way that variably influence the cleavage site specificity, in general favoring cleavage at position 42 relative to that at position 40, thus increasing the Aβ42/40 ratio(Selkoe and Wolfe 2007).

Other than α-, β-, and γ-secretase cleavage, APP also undergoes cleavage at Leu-49 residue of the Aβ sequence this is referred to as the ε-site. This site is C-terminal to the γ-secretase cleavage site. Cleavage at the ε-site, occurs in the late secretory pathway (Weidemann, Eggert et al. 2002). Expression of presenilin-1 mutants decreases the level of ε-cleavage and therefore Aβ production (Weidemann, Eggert et al. 2002). This cleavage is also sensitive to the γ-secretase inhibitors MDL28170 and L-685,458, suggesting that ε-cleavage is also a function of γ-secretase.

In non-neuronal cells, APP is internalized soon after arrival at the cell surface. The YENPTY internalization motif near the C terminus of APP (residues 682–687 of the APP695 isoform) is required for internalization. Following endocytosis, APP is delivered to endosomes. Mutations within the YENPTY endocytosis motif selectively inhibit APP internalization and decrease Aβ generation(Perez, Soriano et al. 1999). The YENPTY and the flanking region serve as the binding site for many cytosolic adaptors that contain phosphotyrosine-binding domains, including Fe65, Fe65L1, Fe65L2, Mint1 (also called X11α), Mint2, Mint3, Dab1, and JNK (c-Jun N-terminal kinase)-interacting protein family members. It has been shown that over-expression of Mint1, Mint2, or Fe65 reduces Aβ generation and deposition in the brains of transgenic mice, strongly suggesting that these adaptors play an important role in regulating APP processing in the nervous tissue(Miller, McLoughlin et al. 2006).Specifically, Fe65 stabilizes the highly

labile AICD, which may serve as a regulatory step in modulating the physiological function of AICD(Thinakaran and Koo 2008).

AICD is produced in both amyloidogenic and non-amyloidogenic processing of APP. There are various AICD isoforms corresponding to different cleavage sites on APP (**Figure 1-2**). All these isoforms contain the YENPTY motif that is required for interaction of AICD with different adaptor proteins. Phosphorylation of AICD has been shown to modify its binding affinity with these adaptor proteins that contain the phosphotyrosine interaction domains (PID) like Fe65 family (Fe65, Fe65L1 and Fe65L2) (Fiore, Zambrano et al. 1995), the X11 family (X11, X11L and X11L2) (Borg, Ooi et al. 1996)etc.The AICD sequence contains eight potential phosphorylation sites. It has been reported that seven of the eight sites(Y653, S655, T668, S675, Y682, T686 and Y687) were phosphorylated in APP from brains of AD patients (Lee, Kao et al. 2003). Phosphorylation of the Y682 residue has been shown to decrease the binding of Fe65 and X11 (Zambrano, Bruni et al. 2001). On the other hand, phosphorylation at T668 is required for Fe65 binding and hence for AICD transcriptional activity (Chang et al. 2006). Fe65 seems to be essential for the nuclear translocation of AICD and variants of AICD mutated at the Fe65 interaction site remains largely cytosolic (Kimberly et al. 2001, Kinoshita et al. 2002a). Fe65 also interacts with a specific histone acetyltransferase, TIP60, and that both these proteins co-localize with AICD in the nucleus and can regulate the transcription of specific genes (Cao and Sudhof 2001). This property of AICD has been utilized to study the production of AICD and therefore as a monitor of APP proteolysis and APP metabolism in a cell culture model (Zhang, Khandelwal et al. 2007)**(Figure 1-3)**.

Expression of several genes can be regulated by AICD. Few of these include KAI1, the prostate cancer anti-metastasis gene; glycogen synthase kinase 3β (GSK3β), involved in tau phosphorylation; BACE and Neprilysin involved with Aβ degradation; p53involved with tumor suppression; HES1 involved with differentiation; and LRP1, EGFR, ACE1, and ACE2 involved with thymidylate synthase (Kim et al., 2003; Sapountzi et al., 2006; Ryan and Pimplikar, 2005; Hebert *et al.*., 2006,reviewed in (Muller, Meyer et al. 2008)).

α-secretase

Processing of APP by α-secretase involves cleavage of APP at Lys16 within the Aβ sequence. This cleavage can occur in the late Golgi compartment (De Strooper, Umans et al. 1993; Kuentzel, Ali et al. 1993) or at the plasma membrane in microdomains known as caveolae (Ikezu, Trapp et al. 1998). As mentioned earlier, α-secretase cleavage of APP leads to the secretion of the soluble fragment sAPPα into the medium in cell culture or is released out of the cell in vivo. Studies have repeatedly shown that hydroxamic acid based active-site directed compounds like batimastat (BB94) and TAPI-2 can inhibit the α–secretase cleavage of APP {(Arribas et al., 1996; Parvathy et al., 1998a; Racchi et al., 1999b) reviewed in (Allinson, Parkin et al. 2003)}. In addition,activation of second messengers, specifically protein kinase C (PKC) with phorbol esters increases α-secretase activity thus upregulating sAPPα secretion, and leading to a significant decrease in Aβ formation (Jacobsen, Spruyt et al. 1994). The different members of α-secretase are ADAM9, ADAM10 and ADAM 17. These are a part of **ad**isintegrin **a**nd **m**etalloproteinase (ADAM) domain family of proteins, which combines features of both cell surface adhesion molecules and proteinases. Like APP,

ADAMs are also type I integral membrane proteins with a multi-domain structure **(Figure 1-4)**. Typically, they consist of an N-terminal signal peptide, followed by a pro-domain containing a "cysteine switch". At the C-terminus of the pro-domain is a cleavage site for the pro-hormone convertases, followed by a catalytic domain containing the HEXXH zinc-binding motif, a cysteine-rich/ disintegrin-/EGF-like domain. This large N-terminal domain is followed by a hydrophobic transmembrane domain, and then a short cytoplasmic domain {reviewed in (Allinson, Parkin et al. 2003)}. ADAM 17 is also known as TNF-α-converting enzyme (TACE; ADAM-17). It is responsible for the release of the inflammatory cytokine tumor necrosis factor (TNF) from its membrane-bound precursor pro-TNF(Black, Rauch et al. 1997). ADAM10 is constitutively expressed in astrocytes in the normal and inflamed human CNS (Kieseier, Pischel et al. 2003), while ADAM10 and ADAM-17 are highly expressed in microglia (Nuttall, Silva et al. 2007). It has been shown that in primary embryonic neurons cultured from TACE knockout mice, there was a deficiency in the phorbol-ester induced release of sAPPα secretion (Buxbaum, Koo et al. 1993) indicating that TACE is indeed an α-secretase. Studies also strongly support the involvement of ADAM10 in the α–secretase cleavage of APP. Studies have shown that ADAM10 protein levels are significantly reduced in the platelets of sporadic AD patients. This correlates with the decrease in sAPPα levels in platelets and cerebrospinal fluid (CSF) of AD patients (Colciaghi, Borroni et al. 2002). A recent study in human glioblastoma cells over-expressing of ADAM9, ADAM10 and ADAM 17 together with a RNAi knockdown of endogenous ADAM9, ADAM10 and ADAM 17 showed that all three enzymes are involved to the same extent in the α-secretase cleavage of APP (Asai, Hattori et al. 2003).

β–secretase

β-Secretase cleavage of APP initiates the amyloidogenic pathway. β-secretase can cleave APP at two sites, the β-site (Met^{671}–Asp^{672}) and the β'-site (Tyr^{681}–Glu^{682}).Cleavage at the β-site is the rate-limiting step in Aβ production, generating a soluble APP fragment (sAPPβ) and two different fragments C99 and C89 (also referred to as CTFβ and CTFβ′) based on the abundance of the enzyme. The C -terminal fragment can be further subjected to the intramembranous γ-secretase cleavage around APP residues 711–713 to produce Aβ. Studies have shown that β-secretase is widely expressed in many tissues but higher level of expression has been observed in the brain (Haass, Schlossmacher et al. 1992; Seubert, Oltersdorf et al. 1993). It is a type-1 transmembrane aspartyl protease, and has been shown to cleave only membrane-bound substrates or substrates closely associated with a membrane-bound protein **(Figure-1-5)**. APP constructs lacking the transmembrane domain are not cleaved in cell culture (Citron, Teplow et al. 1995). The BACE family includes two highly homologous (~60% similarity) type 1 transmembrane aspartyl proteases BACE (β-site APP-cleaving enzyme) 1 and 2 both of which exhibit β-secretase activity. Both BACE1 and BACE2 are transmembrane proteins and can cleave APP at the β-site, but BACE2 preferentially cleaves between amino acids Phe^{690} and Phe^{691} or Phe^{691} and Ala^{692} of APP, within the Aβ sequence (Farzan, Schnitzler et al. 2000). A large body of literature suggests that BACE1 is the major β-secretase responsible for Aβ generation in brain {reviewed in (Vassar 2004)}.

BACE1 contains two active motifs in its luminal domain that harbor the signature sequence of aspartyl proteases (Bennett, Denis et al. 2000). For maturation, nascent

BACE1 is transported through the secretory pathway, subjected to complex glycosylation, and are processed by a furin-like pro-protein convertase (Benjannet, Elagoz et al. 2001; Walter, Fluhrer et al. 2001). BACE1 is found mainly in acidic compartments such as the trans-Golgi network and endosomes (Benjannet, Elagoz et al. 2001). Besides APP, BACE 1can also cleave other proteins. Some of the other targets of BACE1 include sialyl transferase ST6Gal I, the adhesion protein P-selectin glycoprotein ligand-1 (PSGL-1), and APP-like proteins (APLP1 and APLP2) {reviewed in (Westmeyer, Willem et al. 2004)}.

It has been shown that targeted deletion of BACE1 in APP transgenic mice completely abolishes the production and deposition of Aβ(Citron, Teplow et al. 1995), confirming the fact that BACE1 is the only β-secretase. In TG2576 mice that over-express APP, the deletion of BACE gene rescues the Aβ-dependent hippocampal memory deficits (Ohno, Sametsky et al. 2004). Expression of BACE is upregulated in many AD cases. The Swedish APP double mutation, located at -2 and -1 ($Lys^{670} \rightarrow Asn/Met^{671} \rightarrow Leu$) of the β-secretase site enhances the proteolytic efficiency of β-secretase causing a strong increase in Aβ production(Citron, Teplow et al. 1995). BACE cleaves APP with the Swedish FAD-causing mutation (APPswe) 10- to 100-fold more efficiently than wild-type APP.

It has been shown that abolition of BACE expression did not have a drastic effect on model organisms. BACE knockout mice have been shown to be viable and fertile, but have motor neuron myelin defects (Muirhead, Meyerowitz et al. 1992). On the other hand, BACE depletion abolishes Aβ production in APP over-expressing mice (Citron, Teplow et al. 1995). Therefore BACE can be an excellent target for treatment of AD.

Over the past decade several small molecule inhibitors have been developed, that have the ability to cross the blood-brain barrier and also retain the potency to decrease Aβ generation {reviewed in (Silvestri 2009)}. However, the therapeutics are not yet available for routine AD treatment.

γ-secretase

α– and β– Secretase cleavage of APP produces the soluble fragments sAPPα and sAPPβ that are released into the extracellular matrix and the C-terminal fragments CTFα and CTFβ that remain tethered to the membrane. Both CTFα and CTFβ are subsequently cleaved within their transmembrane domain by γ-secretase to produce the short peptide p3 from CTFα, and the full-length Aβ from CTFβ, in addition to the release of the APP intracellular domain (AICD) from both CTFα and CTFβ. γ-secretase is a multimeric complex composed of four proteins: presenilin1 (PS1), Pen2, Aph-1 and nicastrin (NCT) **(Figure 1-6)**. The presence of these four proteins is required for γ-secretase activity.

An early study showed that absence of presenilin1 led to almost complete loss of Aβ peptide generationin neurons (De Strooper, Saftig et al. 1998). This study provided the first clear evidence that presenilins constitute an important part of γ-secretase. Presenilins are aspartyl proteases, where the catalytic site is catalytic site deeply in their large hydrophobic cores (Wolfe, Xia et al. 1999). Presenilins are transmembrane proteins with 8 or 9 transmembrane domains. It can be proteolytically cleaved to produce anamino-terminal (~27- to 30-kDa NTF) and a carboxy-terminal fragment(~16- to 18-kDa CTF) (Spasic, Tolia et al. 2006). There are some studies suggesting that presenilin can form homodimers (Schroeter, Ilagan et al. 2003). Nicastrin is a 130-kDa type I integral membrane protein that is highly glycosylated (Yu, Nishimura et al. 2000). Aph-1

is a seven transmembrane domain protein with the carboxy-terminus located in the cytoplasm (Fortna, Crystal et al. 2004). Pen-2 has a hairpin-like structure with two transmembrane domains and both the amino- and carboxy- termini are located on the lumenal/cytoplasmic side of the domain(Crystal, Morais et al. 2003).

It has been shown that all four γ-secretase proteins co-migrate in non-denaturing electrophoresis gels, and absence of any of the four proteins results in retention of the other components in the endoplasmic reticulumor their rapid degradation and therefore loss of γ-secretase activity, indicating that all the four components for γ-secretase function (De Strooper 2003).

Presenilin cleaves transmembrane domains of many proteins that have a type I conformation of the transmembrane domain (amino terminus oriented to the extra-cellular side of the membrane) and a short (<50 AA) cytoplasmic domain like APP and Notch. In case of Notch, cleavage is triggered by ligand binding, whereas, in case of APP, it is seems to be constitutive. There are two presenilin family members, PS1 and PS2. Mutations in both PS1 and PS2 are associated with familial AD (FAD) (Haass 2004). Both these mutations affect the cleavage specificity of γ-secretase and increase the production of the longer and more neurotoxic form of Aβ, $A\beta_{42}$. The effect of presenilin mutations is the same as that of the FAD mutations reported at the C-terminal fragment of APP (Scheuner, Eckman et al. 1996). Mutations in PS1 seem to be the predominant cause of FAD, as more than 150 FAD mutations have been identified in this gene compared to only few mutations in the PS2 gene (Scheuner, Eckman et al. 1996). Compared to PS1, PS2 has a lower neuronal expression and lower specific activity (Sherrington, Froelich et al. 1996; Bentahir, Nyabi et al. 2006). In the absence of PS1 and

PS2, the production of Aβ is abolished (Herreman, Serneels et al. 2000; Zhang, Nadeau et al. 2000). Thus, presenilins are considered to be the catalytic subunits of the complex.γ-secretase substrates are initially recognized by NCT, which is believed to identify the free N-terminus of a γ-secretase substrate resulting in shedding part of the ectodomain of the substrate (Struhl and Adachi 2000; Shah, Lee et al. 2005). The rest of the substrate is the recognized and bound by PS. It has been reported that G*X*GD active-site motif (Leu^{383} in PS1) is critical for APP/Notch substrate selectivity (Kornilova, Bihel et al. 2005). PEN-2 is required for the stabilization of the PS fragments in the complex (Hasegawa, Sanjo et al. 2004). Aph-1 has been shown to stabilize NCT. The four subunits of γ-secretase assemble into a functional complex in the early compartments of the secretory pathway. In the early compartments of the secretory pathway, NCT and APH-1 form an initial complex, which stabilizes the PS holoprotein. Finally, PEN-2 assembles into this ternary complex and triggers endoproteolysis of PS. When assembly is completed, the complex travels to its functional sites at the plasma membrane and the late compartments of the secretory pathway. Complex formation is tightly regulated and depends on the availability of the individual subunits {reviewed in (Ono, Kanatsu et al. 1989)}.

As mentioned earlier, γ-secretase can cleave a variety of substrates that have a type-I structure. γ-secretase cleavage occurs at different positions in the membrane domain of its substrates, resulting in the generation of a series of small peptides (Aβ,Nβ, etc.), which are secreted at the extracellular side of the membrane, along with a larger polypeptide that is released at the intracellular side (AICD, NICD etc). These fragments have been shown to have transcriptional transactivation properties and can regulate the expression of specific genes. Some of the other functions of γ-secretase include β-catenin

signaling (Kang, Soriano et al. 2002) and Ca2+ signaling etc. {reviewed in (LaFerla 2002)}.

Aβ Metabolism

A large body of literature suggests that the amyloid β-protein (Aβ) plays a central role in Alzheimer's disease (AD). Aβ is derived from the β-amyloid precursor protein (APP) by the action of two aspartyl proteases referred to as β- and γ-secretases. Depending on where γ-secretase cleaves CTFβ, three different forms of Aβ, comprising 38, 40 or 42 amino acid residues, respectively, can produced. The relative amount of $A\beta_{42}$ formed is particularly crucial, because this longer and more neurotoxic form of Aβ is far more prone to oligomerize and form amyloid fibrils than is the shorter, more abundant $A\beta_{40}$ isoform (Burdick, Soreghan et al. 1992; Jarrett, Berger et al. 1993). Aβ is a normally produced in the body (Shoji, Golde et al. 1992)l. 1992), but in some individuals, the over-production of all Aβ species, or an increased proportion of the $A\beta_{42}$ isoform, appears sufficient to cause early onset AD (Rovelet-Lecrux, Hannequin et al. 2006).

Aβ is a natural product and is present in the brains and cerebrospinal fluid (CSF) of normal humans throughout life (Walsh, Tseng et al. 2000). Therefore, the mere presence of Aβ does not cause neurodegeneration. Neurodegenration observed in AD is hypothesized to be a result of synaptic dysfunction caused by hydrophobic aggregates form due to self association of Aβ peptides (Geula, Wu et al. 1998). Within the amyloid plaques that characterize AD, some of the Aβ peptides are organized into 6–10 nm diameter insoluble fibrils, and *in vitro* synthetic Aβ can form amyloid fibrils similar to those present in human brain (Castano, Ghiso et al. 1986; Kirschner, Inouye et al. 1987).

Most studies on AD pathology suggest that aggregation of Aβ is essential for toxicity and neurodegeneration. But in many patients dying with AD, it has been observed that there is a relatively weak correlation between the severity of dementia and the density of fibrillar amyloid plaques and that amyloid plaque number does not correlate well with severity of dementia (Katzman 1986). Some studies suggest a strong correlation between soluble Aβ levels and synaptic plasticity and cognitive impairment (Walsh, Tseng et al. 2000). In a non-AD mouse model, Aβ oligomers have been shown to inhibit long-term potentiation (LTP), enhance long-term depression (LTD) and reduce dendritic spine density (Shankar, Li et al. 2008).

Over a decade the amyloid hypothesis of Alzheimer's disease confirms a central role of Aβ in initiating the AD pathogenic cascade and argues that the neurodegenerative disease process, including the development of neurofibrillary tangles, is a consequence of imbalance between the generation and clearance of Aβ (Hardy and Allsop 1991). Low density lipoprotein receptor-related protein (LRP) mediates the efflux of Aβ from the brain to the periphery (Hammad, Ranganathan et al. 1997). LRP is a multifunctional signaling scavenger receptor that can bind a variety of ligands including apolipoprotein E (apoE), α_2-macroglobulin (α_2M), APP and Aβ (Kang, Pietrzik et al. 2000). LRP binding Aβ mediates the clearance of Aβ. When undergoing LRP-mediated export from the brain, Aβ forms a complex with LRP ligands α2M or apoE on the albuminal side of the endothelium. These complexes bind to LRP, are internalized into late endosomes and are then either delivered to lysosomes, where they are degraded, or undergo transcytosis across the blood brain barrier into the plasma {(Kang, Pietrzik et al. 2000), also reviewed in (Cam and Bu 2006)}. Alternatively, Aβ can also be exported from the brain by directly

binding LRP, although this route of export into the plasma appears to be limited to soluble forms of Aβ {reviewed in (Tanzi and Bertram 2005)}.

In addition to receptor-mediated clearance, Aβ can also be degraded directly by certain proteases. To date, the proteases suggested to play the most important role in proteolyzing Aβ *in vivo* are insulin-degrading enzyme (IDE) and neprilysin {reviewed in (Selkoe 2001)}. IDE degrades a variety of substrates that have the tendency of forming β-pleated sheet (eg. insulin, amylin and Aβ). IDE knockout animals have shown increased levels of cerebral Aβ as well as features of type-2 diabetes (Farris, Mansourian et al. 2004). On the other hand two-fold increases in endogenous IDE levels have been shown to profoundly diminish cerebral Aβ deposition (Leissring, Farris et al. 2003). IDE has also been shown to degrade the AICD, which is thought to play an important role in nuclear signaling and transcriptional regulation *in vitro* and *in vivo* (Edbauer, Willem et al. 2002).

There are several pieces of evidence that suggest that AD pathology might result due to reduced Aβ clearance and not due to increased production of the Aβ peptide. For example the Dutch, Flemish, Italian, and Arctic mutations in the amyloid precursor protein (*APP*) gene encode changes within the sequence of the amyloid beta peptide (Aβ) and cause presenile cerebral amyloid angiopathy, cerebral parenchymal amyloidosis, or both. These disorders are caused by accumulation of Aβ, with no evidence of increased $A\beta_{40}$ or $A\beta_{42}$ production. It actually happens due to changes in catabolism of Aβ in the brain by neprilysin. These mutations thus extend the half-life of Aβ in the brain (Tsubuki, Takaki et al. 2003).

Aβ is degraded in microglia via a pathway sensitive to cytochalasin D and the scavenger receptor inhibitor, fucoidan. ApoE is known to lower the degradation of Aβ (Cole, Beech et al. 1999). As mentioned earlier, Aβ can also be degraded directly by IDE and neprilysin. Other candidate enzymes proposed to regulate Aβ catabolism include angiotensin-converting enzyme (ACE), some matrix metalloproteinases (MMPs), plasmin and, indirectly, thimet oligopeptidase endopeptidase (Carson and Turner 2002). There is some evidence suggesting that Aβ clearance is decreased due to reduced sensitivity to insulin or IGF-I in the brain, as observed in aging, obesity, and diabetes. Such a decrease involves the insulin receptor cascade and can also increase amyloid toxicity. Insulin and IGF-I may modulate brain levels of insulin degrading enzyme, which would also lead to an accumulation of Aβ amyloid (Messier and Teutenberg 2005).

Caloric Restriction, Aging and Neurodegeneration

AD is the major form of dementia with advancing age. However, the molecular events responsible for the development of LOAD have not been clearly defined. Aging can be a reflection of the progressive functional decay of not just one but a complex ensemble of physiological functions. Since the initial study (McCay, Crowell et al. 1989), it has been repeatedly shown that diet strongly influences the incidence and outcome in major age-related diseases including diabetes, obesity and vascular disease. Advances in understanding aging processes and their consequences are leading to the development of therapies to slow down or reverse adverse changes that were initially considered to be “normal” aging and processes that underlie multiple age-related conditions. Since advanced age is the biggest risk factor for late onset AD, studies aimed at investigating the effects of aging therapies like caloric restriction on APP metabolism

has received a lot of attention over the past few years. Chronic caloric restriction (CR) ("under nutrition without malnutrition"), when started in early life or adult life, substantially extends lifespan in rodents as well as in multiple invertebrate species including primates (Roth, Ingram et al. 1999). The fact that this single intervention slows multiple age-related changes, delays the onset of cancer and multiple age related pathologies, and extends life span is consistent with the idea that one of few mechanisms modulated by caloric restriction, control the rate of multiple aging changes and may be potentially controllable by other interventions as well (Hadley, Lakatta et al. 2005). Chronic CR in nonhuman primates has been shown to produce similar physiological changes as those in rodents (Weindruch 1996; Higami, Yamaza et al. 2005). There is also evidence that periodic food deprivation in mice, produced by every other day intermittent feeding, may induce similar physiologic effects over a period of weeks to those of caloric restriction even when average daily intake is different from *ad libitum* intake (Anson, Guo et al. 2003). After a 20-year study it has been very recently reported that CR delayed aging-related deaths in Rhesus monkeys, and also reduced the incidence of diabetes, cancer, cardiovascular disease, and brain atrophy(Colman, Anderson et al. 2009). Caloric restriction has also been shown to improve cellular response to stress in an *in vitro* model of caloric restriction (de Cabo, Furer-Galban et al. 2003). Molecular and physiological mechanisms proposed to explain the effect of CR on lifespan is closely related to the ones that have been proposed to regulate life span by different groups of investigators. They include reduction of oxidative damage, increased metabolic efficiency, decreased apoptosis, lowered glucose levels and most importantly sirtuin activation.

Diet, as mentioned earlier is also a major environmental factor in brain aging. It is now well established, in every species tested to date (yeast, roundworm, rodents, and monkeys), that dietary caloric restriction confers health beneficial effects like slowing down many age dependent processes and extending the lifespan. It is well established that when most types of cells, including neurons, are exposed to a mild stress they increase their ability to resist more severe stress. This" preconditioning" phenomenon involves up-regulation of genes that encode cytoprotective proteins such as heat-shock proteins and growth factors. It has been reported that a similar beneficial cellular stress response can be induced in neurons throughout the brain by a "mealskipping" dietary restriction (DR) regimen in rats and mice. DR is effective in protecting neurons and improving functional outcome in models of stroke, Alzheimer's, Parkinson's and Huntington's diseases. DR induces an increase in the levels of brain-derived neurotrophic factor (BDNF) and heat-shock proteins in neurons. DR also stimulates neurogenesis in the hippocampus, and BDNF plays a role in this effect of DR. Animal studies suggest that it may be possible to reduce the risk for age-related neurodegenerative disorders through dietary and behavioral modifications that act by promoting neuronal plasticity and survival (Mattson et al.,2004). Experimental data suggest that CR regulates adult neuronal stem cells, increases adult neurogenesis in young adult rats (Lee, Seroogy et al. 2002) and reduces age-related declines in neurogenesis in older animals (Bondolfi, Ermini et al. 2004).

Caloric restriction and AD

CR seems to slow down some of the destructive processes that take place in cells and tissues with aging. During the aging process, cells in the brain encounter a cumulative burden of oxidative, metabolic and inflammatory stress associated with numerous modifications of proteins, lipids and nucleic acids (Esiri 2007). All these modifications are exacerbated in neurodegenerative disease; such as Alzheimer's disease where neuronal injury involves brain regions (hippocampus and cortex) involved in learning and memory processes. Over the last few years, numerous studies have undertaken the study of CR on reducing neuronal damage and consequently offer protection against neurodegenerative diseases like AD. CR increases lifespan by slowing many aging processes, including normal aging changes in the brain. In model organisms, CR decelerates the apparent escalation of neuroinflamation and oxidative stress during aging by decreasing both glial activation and production of reactive oxygen species (ROS) (Chong et al., 2005). Short-term caloric restriction in early adulthood attenuated Aβ plaque deposits in transgenic mouse models of AD (Patel et al., 2005). CR also decreased Aβ peptide generation and neuritic plaque deposition in the brain of a mouse model of AD possibly due to promotion of anti-amyloidogenic α-secretase activity (Wang, Ho et al. 2005). Soon after, another group showed that CR resulted in reduced $A\beta_{40}$ and $A\beta_{42}$ peptide contents in the temporal cortex of Squirrel monkeys, compared to (CON) fed monkeys(Qin, Chachich et al. 2006). They also reported that this decrease correlated with an increase in α-secretase active in the brain, which seemed to be due to an increase in SirT1 levels (Qin, Chachich et al. 2006). CR and intermittent fasting (IF) have also been shown to be neuro-protective against the Aβ and Tau induced decrease in

synaptic function in a triple transgenic mouse model of AD (Halagappa, Guo et al. 2007). It has been recently reported that CR attenuates the accumulation of AD-type neuropathology in two cortical brain regions of middle-aged dtg APP/PS1 mice(Mouton, Chachich et al. 2009).

Current AD therapeutics is limited to drugs that treat AD symptoms; so, it is of great interest to identify therapeutics that prevents Aβ-mediated neuronal loss. CR treatment holds considerable potential for identifying novel therapeutic strategies for AD. Currently a large body of research is dedicated to understanding the CR mediated changes in aging, lifespan and also the beneficial effects on neurodegenerative diseases like AD.

Caloric Restriction and SirT1

In diverse organisms, caloric restriction slows the pace of aging and increases maximum lifespan. Caloric restriction extends life span by increasing the activity of Sir2 (Silencing Information Regulator), a member of the conserved Sirtuin family of NAD^+-dependent protein deacetylases (Howitz, Bitterman et al. 2003; Denu 2005). SIR-2.1 enzyme in *C.elegans* that regulates lifespan, and SirT1, is the human orthologue that promotes cell survival, belong to the same family. The Sir2 family of protein deacetylases is involved in NAD (Nicotinamide Adenine Dinucleotide)-dependent nuclear enzymatic activities. Sir2 is an NAD-dependent deacetylase of histones and other proteins and its activation is central to promoting increased longevity in yeast and C.elegans (Howitz, Bitterman et al. 2003).

Sir2 proteins are effectors of the axonal protection mediated by increased Nmnat activity. Neurons when treated with resveratrol (a poly-phenol compound found in grapes, that

induces the activity of Sir2, by lowering its Km), there is a substantial decrease in axonal degradation. The induction of Sir 2 by resveratrol also depends on the availability of NAD (Araki, Sasaki et al. 2004). In humans and rodents, there are seven genes that share the Sir2 conserved domain [sirtuin (SIRT) 1 to 7]. Out of those, only SirT1 is located in the nucleus and is involved in chromatin remodeling and the regulation of transcription factors such as p53 (Chen, Wang et al. 2005), where as other SIRT proteins are located within the cytoplasm and the mitochondria. SirT1 is the major effector of the increased NAD supply that effectively prevents axonal self-destruction.

Resveratrol lowers the Michaelis constant of SirT1 for both the acetylated substrate and NAD+, and increases cell survival by stimulating SirT1-dependent deacetylation of p53 (Chen, Wang et al. 2005). This suggests that that the alteration of NAD levels by manipulation of the NAD biosynthetic pathway, sirtuin activity or other downstream effectors might provide new therapeutic opportunities for the treatment of diseases involving axonopathy and neurodegeneration. However, the molecular mechanism of activation of SirT1 by resveratrol is still not well understood. Resveratrol treatment mimics the effects of caloric restriction, which has been demonstrated by the fact that it helps increase the average lifespan of yeast by 70%. The ability of resveratrol to induce SirT1*in vivo* has been studied by Sinclair et al. in 2003(Howitz, Bitterman et al. 2003). SirT1 promotes cell survival by negatively regulating the tumor suppressor p53 (Vaziri, Dessain et al. 2001). One known target of SirT1 is Lysine 382 (K382) of p53. Deacetylation of this p53 residue by SirT1 decreases the activity and half-life of p53, and increases cell survival under a variety of DNA damaging conditions (Appella and Anderson 2001; Brooks and Gu 2003). Resveratrol treated cells show a substantial

decrease in the level of Ac-K382. This clearly indicates that resveratrol promotes cell survival by stimulating SirT1*in vivo*. In this property, resveratrol is thought to mimic caloric restriction. Since survival and longevity at the cellular and molecular level are intimately linked, the ability of polyphenols to promote cell survival by activating Sirtuins calls for a new line of investigation on the effects of these and related molecules on age-related diseases, including AD.

Caloric restriction induces SirT1 expression in a wide array of tissues, and this shifts the balance away from cell death toward cell survival. A critical step in initiating stress-induced apoptosis is the relocalization of Bax protein from the cytoplasm to the outer mitochondrial membrane and the subsequent release of cytochrome *c*. Under normal conditions, Bax is rendered inactive in the cytoplasm by its tight association with Ku70, a DNA repair factor. In response to stress, two critical lysine residues in Ku70, K539 and K542 become acetylated by acetyltransferases, and the Ku70-Bax interaction is disrupted. This allows Bax to localize in the mitochondria and initiate apoptosis (Cohen, Lavu et al. 2004). In response to caloric restriction, SirT1 deactylates the DNA repair factor Ku70. It maintains K539 and K542 of Ku70 in a deacetylated state to keep Bax away from the mitochondria (Cohen, Lavu et al. 2004). In addition to the Ku70-Bax pathway, numerous other SirT1 targets are presumably affected by caloric restriction, including p53.

Mammalian SirT1 also appears to control cellular response to stress by regulating FOXO transcription factors. FOXO are a family of protein that functions as sensors to the insulin signaling pathway and as regulators of organismal longevity. In mammalian cells, in response to oxidative stress, especially H_2O_2 and also heat shock, SirT1 deacetylase

forms a protein complex with the Forkhead transcription factor FOXO3 that contributes to deacetylation of FOXO3. SirT1 differentially affects the function of FOXO3, pertaining FOXO3's effect on cell cycle arrest and DNA repair target genes, but attenuating FOXO3 dependent apoptosis in the presence of stress stimuli (Brunet, Sweeney et al. 2004). Under cellular stress, SirT1 deacetylation of p53 also leads to inhibition of apoptosis. Since SirT1 also reduces FOXO3 induced apoptosis in the presence of stress stimuli, there might exist some interaction between FOXO3 and p53, in terms of mediating the effects of SirT1. In another group of studies, it has been shown by Finkel et al. (2004) that the interaction between wild-type FOXO3 and p53 is strongly dependent on nutrient availability. Under normal nutrient conditions, the predominant effect of p53 involves repression of SirT1. In contrast, under starved conditions, the ability of activated FOXO3a to stimulate SirT1 expression requires p53(Nemoto, Fergusson et al. 2004). This suggests that in absence of p53, the basal expression level of SirT1 might rise but the starvation-induced increase would not be sharp.

It has been reported that caloric restriction also extends lifespan by modulating glucose metabolism, which again involves SirT1. SirT1 regulates the gluconeogenic/glycolytic pathways in liver in response to fasting signals through the transcriptional co-activator PGC-1α. A nutrient signaling response that is mediated by pyruvate induces SirT1 protein in liver during fasting. Once SirT1 is induced, it interacts with and deacetylates PGC-1α at specific lysine residues in an NAD^+ dependent manner (Rodgers, Lerin et al. 2005). So it can be concluded that SirT1 is involved in the CR mediated affects on lifespan in different model organisms.

SirT1 and AD

AD is a neurodenerative disorder and age is the single most important risk factor for developing late-onset Alzheimer's disease (AD) (Terry 2006; Turner 2006). AD research over the last few years have shown that numerous related mechanisms, such as accumulation of toxic proteins, ubiquitin-proteasome system dysfunction, excitotoxic insult, oxidative stress, mitochondrial injury, synaptic malfunction, altered metal homeostasis and disruption of axonal and dendritic transport (Bossy-Wetzel, Schwarzenbacher et al. 2004; Selkoe 2004), lead to neurodegeneration. Numerous studies in model organisms have shown that Sir2/SirT1is a critical regulator of the aging process (Anderson, Bitterman et al. 2003; Howitz, Bitterman et al. 2003; Cohen, Miller et al. 2004). The beneficial effect of SirT1 on the nervous system has been shown by many groups. For example, knockdown of the *SIRT1* gene in cultured mouse dorsal roots ganglion sensory neurons reverses the protective effects of increased NAD^+ synthesis on axonal degeneration following acute axotomy (Araki, Sasaki et al. 2004). Both genetic and pharmacologic induction of SirT1 has also shown to be beneficial in many *in vivo* and *in vitro* models of AD. The SirT1 agonist resveratol has been shown to lower the levels of secreted and intracellular Aβ peptides produced from different cell lines by a mechanism that induces degradation of Aβpeptides via the proteasomal pathway (Marambaud, Zhao et al. 2005). It has been recently shown that SirT1 mediates the CR mediated effects in brains of Tg2576 mice. It has been further described that this effect of SirT1 is mediated by increasing the non-amyloidogenic processing of APP (Qin, Yang et al. 2006). In p25 transgenic mouse model of AD, resveratrol reduced neurodegeneration

in the hippocampus, improved learning ability, and decreased the acetylation of the known SIRT1 substrates PGC-1α and p53 elucidating another possible mechanism of SirT1 action in the nervous system(Kim, Nguyen et al. 2007). Oral administration of resveratrol for 45 days in transgenic mouse model of AD decreases plaque formation (Karuppagounder, Pinto et al. 2009).

These studies highlight that CR via SirT1 has beneficial effect on the molecular and pathological features of AD.

Summary of Dissertation Research

Since age is the biggest risk factor for AD, I hypothesized that "**the molecular pathways that modulate aging/lifespan, must modulate the development of AD**". As I discussed before CR is known to modulate lifespan in different model organisms. But, to specifically study the effect of CR on APP metabolism, I started off with using an in vitro model of CR, previously established by de Cabo et al in 2003, which has been shown to recapitulate some of the effects of *in vivo* caloric restriction, such as increased resistance to stressors like heat shock and H_2O_2 (de Cabo, Furer-Galban et al. 2003) and increased levels of SirT1 (Cohen, Miller et al. 2004).. This *in vitro* model which relies on cultures of human cell lines in media supplemented with 10% sera obtained from rats fed *ad libitum* (AL) or caloric restriction (CR) regimens (de Cabo, Furer-Galban et al. 2003; Cohen, Miller et al. 2004).

We studied the effect of *in vitro* CR on APP metabolism utilizing naïve or APP-Gal4 and/or Swedish APP_{695} over-expressing SH-SY5Y and HEK-293 cells, respectively. We showed that *in vitro* CR decreases secreted and intracellular Aβ levels, AICD mediated transactivation (Zhang, Khandelwal et al. 2007) and also full-length APP

levels. These latter effects on full-length APP were not due to changes in APP transcription. The previously described model of *in vitro* CR utilized serum from AL and CR fed F344 rats. We further tested the effect of CR utilizing FBN rat serum, to determine if the effect of *in vitro* CR that we observed so far were rat strain specific. We showed here for that first time that using serum from AL and CR fed FBN rats for this *in vitro* model also decrease AICD mediated transactivation and full-length APP levels, indicating that our observed effects of *in vitro* CR on APP metabolism were not dependent on the type of rat serum used.

Next, we wanted to study the mechanism of these *in vitro* CR mediated changes on APP metabolism. AS I discussed earlier, the NAD-dependent deacetylase SirT1 has been most commonly associated with the CR mediated changes in longevity and aging process in different model organisms. So, we specifically, we wanted to determine if effects of *in vitro* CR on APP metabolism are via SirT1. Exposure of the aforementioned cell lines to *in vitro* CR conditions resulted in increased SirT1 levels. Therefore, we wanted to study if SirT1 over-expression is able to recapitulate the effects of *in vitro* CR on APP metabolism. So, we performed the similar line of experiments and observed that some of the effects of *in vitro* CR were recapitulated upon over-expressing SirT1 or inducing its activity with resveratrol. Our results demonstrate that SirT1 modulates APP metabolism. Specifically, SirT1-over-expression decreases AICD levels, but does not affect full-length APP levels. We also observed that SirT1 increases CTFα and sAPPα levels indicating that it induces non-amyloidogenic processing of APP in these cell lines.

AD is a disease of the human central nervous system, so it was important to study if the effects of CR and SirT1 that we observed in our cell culture model can be recapitulated

in an *in vivo* setting. To determine if CR and/or SirT1 can modulate APP metabolism *in vivo,* we utilized an AICD dependent apoptosis reporter system in the *Drosophila*eye (Guo, Hong et al. 2003; Greeve, Kretzschmar et al. 2004; Gross, Feldman et al. 2008). These flies were (i) raised in conditions of CR or AL, or (ii) genetically manipulated to have Sir2 either over-expressed or inactivated. We demonstrated that CR exposure of these flies suppress the AD phenotype. Furthermore, we demonstrate for the first time that Sir2 over-expression, as well as exposure to resveratrol, in these flies suppresses the AD phenotype, while Sir2 inactivation enhances this phenotype.

The *Drosophila* model that we used for this study expresses the C-terminal fragment of APP (Guo, Hong et al. 2003). In fact, all previously described *Drosophila* models of AD rely either on expression the of the toxic $A\beta_{42}$ peptide in the nervous system(Iijima, Liu et al. 2004; Iijima, Chiang et al. 2008), or expression of the human APP and β-secretase (BACE) ubiquitously in all tissues, or in the developing retina(Greeve, Kretzschmar et al. 2004) or wing(Fossgreen, Bruckner et al. 1998). Therefore, to study the effect of different genetic (like *SirT1*) and pharmacologic (e.g. resveratrol) modulators of APP metabolism, we felt the need of establishing a *Drosophila* model that would allow the natural processing of APP by β– and γ–secretase in the central nervous system.

To establish our model, we expressed the human forms of APP and BACE in flies using the Gal4/UAS system {described (Brand and Perrimon 1993)}. By expressing the human forms of APP and BACE, we require that the normal cleavage events must occur to generate toxic Aβ peptides within the fly. Further, we utilized the *ELAV-Gal4* reagent to limit expression of these proteins to the fly nervous system only. We believe that

through this specific experimental protocol, we are creating a situation that is more similar to that which is observed in human AD patients. We initially tested for successful expression of these proteins in adult fly heads through Western Blot analysis. We observe strong expression of APP in both APP only and APP, BACE over-expressing flies, upon induction with ELAV-Gal4.CTFα (C83) was mostly detected in APP over-expressing flies induced with ELAV-Gal4 (ELAV-Gal4/UAS APP flies). On the other hand CTFβ (C99) was mostly detected in APP and BACE over-expressing flies induced with ELAV-Gal4 (ELAV-Gal4/APP; BACE flies) indicating that presence of BACE in these flies drives the APP proteolysis through the non-amyloidogenic pathway. To determine if the observed changes in Drosophila phenotypes are dependent on γ–secretase cleavage of APP, we raised these AD model flies on food containing L-685, 458, a potent γ–secretase inhibitor. In ELAV-Gal4/APP flies, L-685, 458 exposure led to a buildup of the C83 fragment. On the other hand in ELAV-Gal4/APP; BACE flies, L-685, 458, exposure led to a buildup of the C99 and C83. Our AD model flies displayed 6E10 (β-amyloid specific antibody) positive amyloid plaques and Thioflavin S positive puncta in the brains indicating that these flies can generate Aβ peptides through β- and γ-secretase processing of APP and develop the stereotypical AD neuropathology. We also studied the neuroanatomical structures of our AD model flies and showed that flies expressing both APP and BACE show massive neurodegenration in parts of the brain involved in learning and memory, similar to AD patients. As expected, these molecular and neuropathologic effects are rescued by treatment γ-secretase inhibitor L-685,458. Finally, we studied longevity and CNS function, as assessed by climbing behavior, of these flies and showed that the AD model flies expressing both APP and BACE had significantly decreased

longevity and climbing ability compared to control flies that was rescued by L-685,458. We also tested the effect of the Sir2 agonist resveratrol on the climbing behavior of our AD model flies and have shown that resveratrol improves the climbing ability in these flies. Therefore this model will serve as a powerful tool for future screening of genetic and pharmacologic modulators of APP proteolysis and Aβ production.

Figures

Figure 1-1 Intracellular trafficking of APP(Thinakaran and Koo 2008)

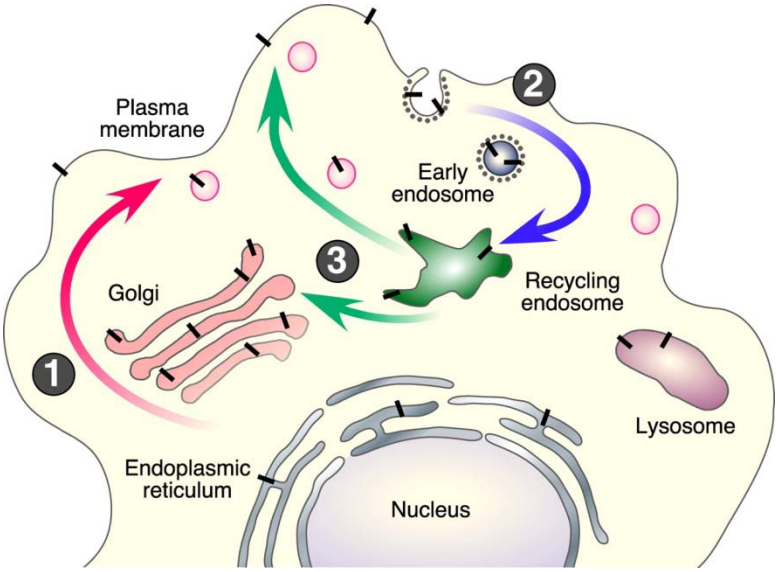


Figure 1-2. A schematic diagram of amyloid β precursor protein (APP) proteolytic processing(Wakabayashi and De Strooper 2008)

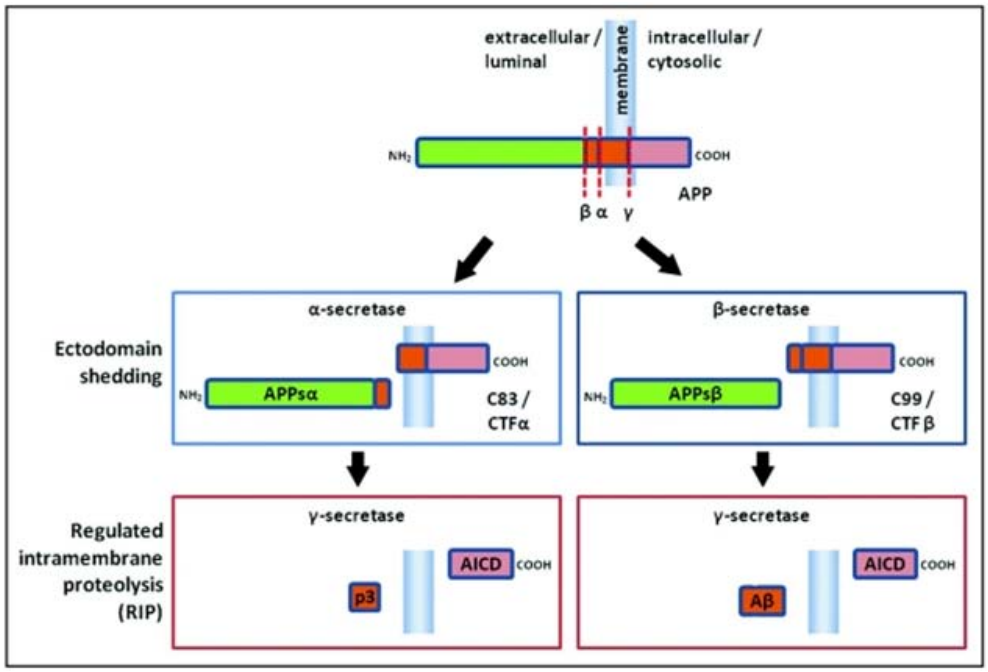


Figure 1-3. APP transactivation assay

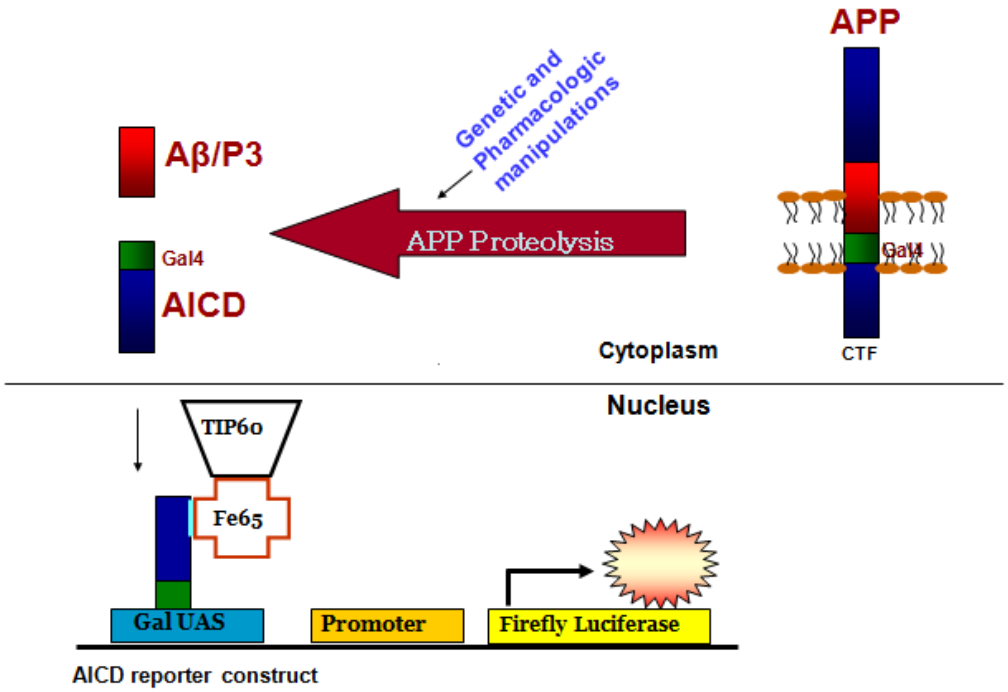


Figure 1-4. Domain structure of α–secretase (Allinson, Parkin et al. 2003)

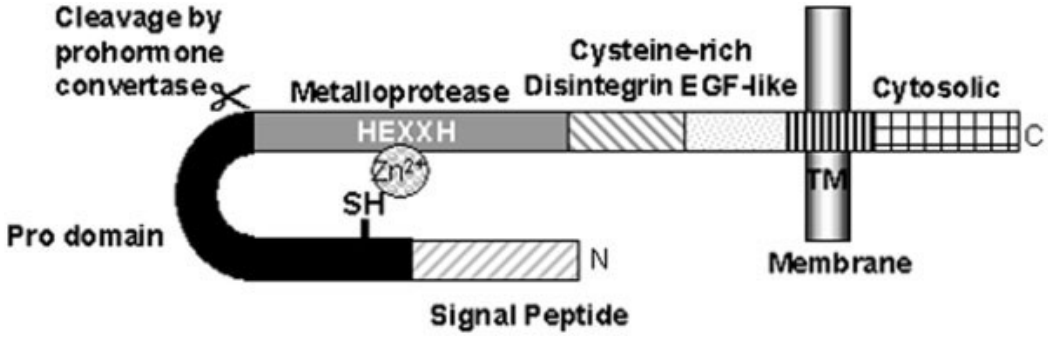


Figure 1-5. Structural organization of BACE1 (Vassar, Kovacs et al. 2009)

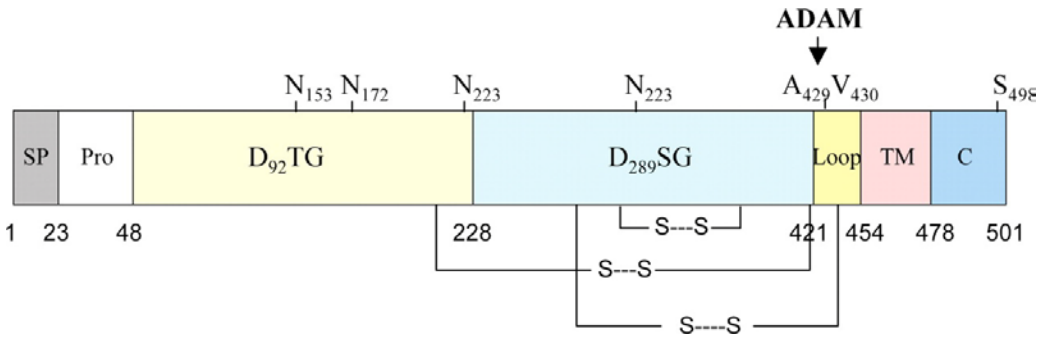
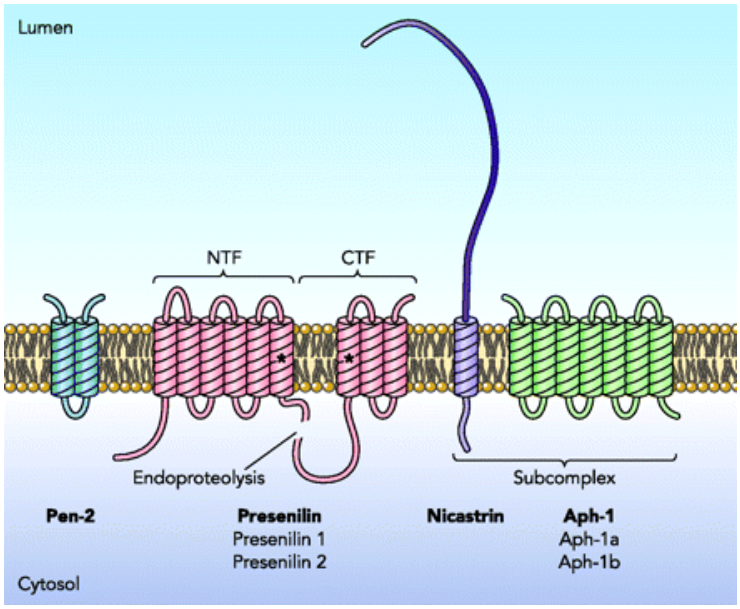


Figure 1-6. γ–Secretase components (Wakabayashi and De Strooper 2008)



CHAPTER 2

In vitro and *in vivo* caloric restriction modulates APP metabolism

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Running title: SirT1 Modulates APP metabolism *in vitro and in vivo*

Abstract

Caloric Restriction (CR) "slows" aging by increasing the survival of critical cell types against a variety of stressors. Advanced age is the largest risk factor for developing Alzheimer's disease (AD). The molecular events that lead to AD-related neuronal loss are dependent on proteolytic processing of the amyloid precursor protein (APP) which generates the neurotoxic Aβ peptide. Utilizing an established cell culture model that recapitulates the major cellular phenotypes of CR, we have investigated whether *in vitro* CR modulates the production of Aβ. We find that in this *in vitro* CR model, Aβ levels are decreased and APP metabolism is altered. Exposure of human cell lines to *in vitro* CR conditions results in decreased full-length APP levels and increased sAPPα and SirT1 levels. SirT1 over-expression also recapitulates some of the effects of CR. To determine if CR and SirT1 modulate APP metabolism *in vivo,* we utilized an established Drosophila model of AD. We show that CR decreases the AD phenotype in these flies. Sir2 gain-of-function alone recapitulates the phenotypic effect of CR, while Sir2 loss-of-function mutations enhance the fly AD phenotype. These *in vivo* findings are in accord with our *in vitro* finding and add to the growing evidence that both nutritional status and SirT1 can regulate APP metabolism and can modulate the risk for developing AD.

Key words: SH-SY5Y, HEK-293, *Drosophila melanogaster,* Aβ, caloric restriction

Introduction

Age is the single most important risk factor for developing late-onset Alzheimer's disease (AD) (Terry 2006; Turner 2006). The frequency of Alzheimer's disease has increased steadily with increases in life expectancy (Evans, Funkenstein et al. 1989). By the mid-century, the number of people living with AD is expected to quadruple(Brookmeyer R. 2007). However, the molecular events responsible for the development of late-onset Alzheimer's disease (LOAD) have not been clearly defined. Advances in understanding the molecular pathways that control the aging process are leading to the development of therapies to slow down or reverse "normal" aging as well as age-related diseases, such as AD.

Diet strongly influences the incidence and outcome of age-related diseases including diabetes, obesity and vascular disease (Morgan, Wong et al. 2007). Caloric restriction (CR) is the only known non-genetic way to extend lifespan and delay/stop aging-related cellular dysfunctions in mammals (Messier and Teutenberg 2005). Caloric restriction (a 40% decrease in total calorie intake) is an intervention shown to increase the average and maximum lifespan in model organisms. In rodents, age-related increases in neuroinflammation and oxidative stress markers are suppressed by a CR dietary regimen (Chong, Lin et al. 2005). In addition, CR suppresses the age-related decline in hippocampal-dependent cognitive function (Martin, Mattson et al. 2006; Shi, Adams et al. 2007). It has been recently reported that CR also reduces the incidence of age-related diseases and mortality in Rhesus monkeys (Colman, Anderson et al. 2009).

In model organisms such as *Saccharomyces cerevisae*, *Caenorhabditis elegans* and*Drosophila melanogaster* the life-extension effects of CR observed are dependent on the activity of Sir2 (Silencing Information Regulator), a member of the conserved Sirtuin family of NAD-dependent protein deacetylases (Howitz, Bitterman et al. 2003; Partridge, Piper et al. 2005; Lee, Wilson et al. 2006). *Sir2* encodes an NAD+ (nicotinamide adenine dinucleotide) -dependent deacetylase of histones and other proteins. It promotes cell survival by deacetylating p53 and FOXO3(Vaziri, Dessain et al. 2001; Smith 2002; Brunet, Sweeney et al. 2004; Giannakou and Partridge 2004). In *Saccharomyces cerevisae*, Sir2 has been shown to extend replicative lifespan by reducing the formation of extrachrosomal rDNA circles (Denu 2005).

In humans and rodents, there are seven genes that share the Sir2 conserved domain [sirtuin (SIRT) 1 to 7]. Out of those, only the SirT1 localizes to the nucleus where it is involved in chromatin remodeling (Chen, Wang et al. 2005). Caloric restriction induces SirT1 expression in a wide array of tissues and results in suppression of cell death and an enhancement of cell survival (Qin, Yang et al. 2006). SirT1 negatively regulates p53 activity and decreases p53 half-life by deacetylating lysine 382 of p53. This results in a cell survival increase in response to a variety of DNA damaging conditions(Vaziri, Dessain et al. 2001; Smith 2002; Chen, Wang et al. 2005). Similarly, SIRT1 deacetylates the Fork-head transcription factor, FOXO3, resulting in resistance to oxidative stress and reduced apoptosis (Giannakou and Partridge 2004). SirT1 activity also mediates changes in somatotropic signaling and physical activity that are associated with caloric restriction (Cohen, Supinski et al. 2009)

Alzheimer's disease is the major form of dementia in U.S and the symptoms of the disease are caused by massive loss of synapses and neurons primarily in the cerebral cortex and hippocampus (Kril, Patel et al. 2002). The β-amyloid peptides (Aβ) play a fundamental role in triggering this synaptic and neuronal loss, which eventually lead to cognitive decline(Selkoe 2002).

Aβ peptides are generated by proteolytic processing of the β-amyloid precursor protein (APP) (Annaert and De Strooper 2002). APP is a type-I transmembrane protein with a relatively large extracellular domain and a small intracellular domain. Aβ peptide generation is initiated when APP is cleaved by the aspartyl protease BACE (β-site APP Cleaving Enzyme) at the N-terminus of the Aβ sequence producing a soluble N-terminal fragment, termed sAPPβ, and a C-terminal transmembrane fragment, termed CTF-β or C99(Vassar, Bennett et al. 1999). The C99 fragment is further cleaved by the γ-secretase protease complex at the C-terminus of the Aβ sequence to produce Aβ and the APP Intracellular Domain (AICD)(De Strooper and Annaert 2000). AICD has been implicated in nuclear signaling (Cao and Sudhof 2001). Aβ peptides can oligomerize and eventually form large aggregates which are the main components of cerebral amyloid plaques, the central pathological feature of AD (Hardy and Selkoe 2002; Tanzi and Bertram 2005). Small molecular weight Aβ oligomers can modulate synaptic activity (Selkoe 2002). Aβ also has neurotoxic properties that can lead to neuron death (Ueda, Fukui et al. 1994). Taken together Aβ peptide accumulation seems to be responsible for the neuronal dysfunction and neuronal loss that defines AD.

An alternative mechanism of APP proteolytic processing occurs in non-neuronal cells, where initial APP cleavage is performed by α-secretase.α-secretases (ADAMs 9, 10,

and 17) cleave APP between Lys16 and Leu 17 of the Aβ sequence (residues 612 and 613 of APP_{695}) and therefore precludes the generation of Aβ peptide. This cleavage produces a soluble N-terminal fragment, termed sAPPα, and a C-terminal transmembrane fragment, termed CTFα or C83. The C83 fragment is then cleaved by γ-secretase protease complex. Cleavage occurs at the C-terminus of the Aβ sequence resulting in the production of a non-amyloidogenic peptide (P3) and AICD.

Current AD therapeutics are limited to treating AD symptoms; so, it is of great interest to identify therapeutics that prevents Aβ-mediated neuronal loss. CR treatment holds considerable potential for identifying novel therapeutic strategies for AD. In transgenic mouse models of AD, CR reduces the severity of symptoms. Specifically, the cerebral Aβ plaque deposition that is typical in these models is significantly decreased after short term CR (Patel, Gordon et al. 2005; Wang, Ho et al. 2005). Studies on AD mouse models suggest that SirT1 may help to mediate decreases in Aβ levels after CR treatment (Qin, Yang et al. 2006). There have been a number of *in vitro* approaches aimed at studying the effects of caloric restriction on pathways that regulate aging. Most of these studies have utilized primary cell cultures from animal models (Pignolo, Masoro et al. 1992; Li, Yan et al. 1997; Lambert and Merry 2000; Qin, Yang et al. 2006).

In our present study we have utilized an *in vitro* model of caloric restriction which relies on cultures of human cell lines in media supplemented with sera obtained from rats fed ad libitum (AL) or caloric restriction (CR) regimens (de Cabo, Furer-Galban et al. 2003; Cohen, Miller et al. 2004). This established model recapitulates some of the major effects of CR seen in animals(de Cabo, Furer-Galban et al. 2003). One of the advantages of

this approach is that it allows for simpler experimental manipulation by genetic and pharmacologic methods, than those compared to animal CR models (de Cabo, Furer-Galban et al. 2003; Cohen, Miller et al. 2004).

Here we show for the first time that *in vitro* caloric restriction decreases the steady state levels of secreted Aβ, AICD levels and AICD mediated transactivation, C83 and full-length APP levels in SH-SY5Y (human neuroblastoma cells) and HEK-293 (human embryonic kidney) cell lines. We find that these *in vitro* CR mediated effects coincide with increased SirT1 levels, as observed in other CR models. We can recapitulate some of the effects of *in vitro* CR on APP metabolism by over-expressing SirT1 or by treating with the SirT1 agonist resveratrol, suggesting that SirT1 plays a significant role in these effects; while other effects can be recapitulated by over-expressing PGC-1α. To determine if CR or SirT1 can modulate APP metabolism *in vivo*, we utilize an AICD dependent reporter system in *Drosophila*(Guo, Hong et al. 2003; Greeve, Kretzschmar et al. 2004; Gross, Feldman et al. 2008). Flies raised on calorically restricted diets had a suppressed AICD-dependent phenotype compared to flies raised on *ad libitum* diets. When raised on normal diets over-expression of Sir2 or exposure to resveratrol also suppressed the AICD-dependent phenotype. In contrast, Sir2 inactivation enhanced this phenotype.

Materials and Methods

Cell Culture and treatments: HEK-293 cells (obtained from ATCC) were used for studying the effects of various treatments on endogenous levels of APP and its proteolytic fragments. HEK-293 cells were transiently transfected with pBABE puro^r empty vector, pBABE SirT1 over-expression plasmid, (generously provided by Dr. D. Sinclair). HEK-293 cells were cultured in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml) and streptomycin (25,000 μg/ml). SH-SY5Y cells (obtained from ATCC) were cultured in DMEM, supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml) and streptomycin (25,000 μg/ml). SH-SY5Y cells stably expressing APP Gal4 and Gal4 UAS reporter construct (SY5Y-APP Gal4 cells) and HEK-293 cells stably transfected with Swedish APP_{695} plasmid (APPSwed-293 cells) were cultured in DMEM, supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml), streptomycin (25,000 μg/ml) and G418 (200 μg/ml). For the caloric restriction (CR) and *ad libitum* treatments (AL), media were prepared by supplementing 1x DMEM with AL or CR serum obtained from NIA Rodent Tissue Bank (de Cabo, Furer-Galban et al. 2003). AL serum was collected from 28 month/18 month old NIH Fisher 344 rats and 34 month old NIH Fisher Brown Norwegian (FBN) rats that were fed a NIH-31 standard diet. CR serum was collected from 28 month/18 month old NIH Fisher 344 rats and 34 month old NIH Fisher Brown Norwegian (FBN) rats that were given a vitamin and mineral fortified version of the AL diet. CR animals were in a 60% calorie restriction since weaning. Water was available *ad libitum* for both groups. Conditions in which the animals were maintained have been

described in another study (de Cabo, Furer-Galban et al. 2003). For our studies HEK-293 and/or APP Gal4 SH-SY5Y cells were treated with 1x DMEM supplemented with either AL or CR rat serum, for 48 hours.

Plasmids: For the APP transactivation assay, APP Gal4 fusion plasmid was utilized (Zhang, Khandelwal et al. 2007). For SirT1 over-expression experiments, empty pBABE vector with a puromycin resistant gene, pBABE puro^r vector with SirT1 over-expression and pBABE puro^r vector with SirT1ΔHY (dominant negative SirT1 gene) (Cohen, Miller et al. 2004)were used. These plasmids were a gift from Dr. D. Sinclair's lab. For SirT1 over-expression experiments we used HEK-293 cells stably transfected with Swedish APP_{695} plasmid.

APP transactivation assay: For the transactivation assay, SH-SY5Y cells were stably transfected with pMSt APP (APP Gal4) construct and pG5e1B-luc (Gal4 reporter plasmid) and the neomycin (G418) resistant plasmid pcDNA3.1 (Invitrogen) (Zhang, Khandelwal et al. 2007). The cells were maintained in DMEM, supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml), streptomycin (25,000 μg/ml) and G418 (200 μg/ml). After CR/AL treatments in 24/96 well plates, the cells were washed twice with cold PBS and then lysed with GLB (Glo Lysis Buffer, Promega Inc.). Then Luciferase activity was measured using Steady Glo luciferase reagent, Promega Inc. The luciferase activity was measured using the TopCount plate reader (PerkinElmer.Inc). The luciferase counts per second (CPS) were normalized to cell count by Sybergreen Assay. For Syber green assay, 20x Syber Green (diluted with PBS from 1000x stock) was used.

Experiments were done in triplicates. For the Dual luciferase assay, Renilla luciferase plasmid pRLSV40 (Promega) is transiently transfected in SH-SY5Y cells stably transfected with pMSt APP (APP Gal4) construct and pG5e1B-luc (Gal4 reporter plasmid). Dual luciferase assay is also performed by transiently co-transfecting HEK-293 cells with pMSt APP (APP Gal4) construct andpG5e1B-luc (Gal4 reporter plasmid) plasmids with one-tenth of Renilla luciferase pRLSV40 (Promega) reporter construct. Both cell types are transfected with Arrestin (Open Biosystems). For the Dual Luciferase assay cells were washed twice with cold PBS and lysed with Dulbecco's modified Eagles medium, supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml),streptomycin (25,000 μg/ml) and G418 (200 μg/ml). Measurement of firefly luciferase activity (dependent on APP processing) and Renilla luciferase activity (independent of APP processing) are done using the Dual Glo kit (Promega). In the case of the Dual Luciferase Assay, the firefly luciferase counts were normalized to Renilla luciferase counts.

Western Blot Analysis: After the treatments, cells were washed twice with cold PBS and then lysed in RIPA Buffer containing different protease inhibitors [Antipain (100 μM), Aprotinin (2 μg/ml), Benzamide (15 μg/ml), Chymostatin (100 μM), Leupeptin (100 μM), Pepstatin A (1 μM), PMSF (1 μM), Sodium Metabisulfite(0.1 nM). 10μl of lysates were used for protein assay with the help of BCA Protein Assay Kit (Pierce, Inc.). The BCA protein utilizes a standard curve generated by RIPA standards (different dilutions of BSA protein in RIPA buffer). According to the protein concentrations, samples for Western Blot were prepared using the 4x Nupage LDS

sample buffer (Invitrogen, Inc.) containing 0.2% BME (β-Mercaptoethanol, Sigma Aldrich). Equal volumes of protein were loaded on to each well of NuPAGE 4-12% Bis Tris Gel. From the gel the proteins were transferred on to 0.25 μm PVDF membrane (Millipore) using a semi-dry transfer apparatus. Blots were probed with different antibodies and the target protein densitometry was normalized to actin densitometry using a Fluorochem 8900 gel imaging system (Alpha Innotech) and/or Odyssey Infrared Imaging system (LI-COR Biosciences).

TCA precipitation for quantification of sAPPα levels: Following transient transfection with SirT1, SY5Y-APP Gal4 cells were exposed to serum free media for 2 hours after which the conditioned media was collected. SY5Y-APP Gal4 cells were also treated with 50 μM resveratrol or vehicle control (DMSO) diluted in serum free media for 4 hours. Following these treatments the conditioned media was collected. From both the experiments, 1 ml of conditioned media from each sample was precipitated with 338 μl of TCA. After 15-60 minutes incubation, tubes were spun at 14,000 rpm for 15 minutes. Clear top layer was removed from each tube and 500 μl of Acetone was added. At this point samples were frozen over night. Then they were spun at 14,000 rpm for 15 minutes in ice-cold condition. Supernatant was removed and pellet was dissolved in 20 μl of 1x Protein loading Dye. This entire volume was used for Western Blot.

Antibodies: We utilized the following primary antibodies: a polyclonal antibody raised to the C-terminus of APP (A8717; Sigma Aldrich, Inc), a monoclonal anti-β-Actin antibody (A5441, Sigma Aldrich, Inc), a monoclonal anti-SirT1 antibody (05-707,

Upstate, Inc), a monoclonal antibody (6E10) to the Aβ region of APP (ab10146, Abcam). For ECL based western blot detection we utilized the following secondary antibodies: goat anti-rabbit (NA934V, GE Healthcare UK Limited) and goat anti-mouse (NA931V, GE Healthcare UK Limited). For Western blot detection using the Odyssey Infrared Imaging system we used the following secondary antibodies: goat anti-Rabbit IR-Dye800 CW (926-3211) and/or Goat anti-Mouse IR Dye 680 (926-3200).

***Aβ ELISA*:** Amyloid beta (1-40) ELISA Kit (Biosource) was used for our experiments. After the CR or AL treatment, the samples were diluted in 1x DMEM (1:2). Both the conditioned media and the lysates were diluted the same way. Conditioned media was used for measuring secreted $A\beta_{40}$ and lysates were used for measuring intracellular $A\beta_{40}$.

Pharmacologic reagents used: Resveratrol (Sigma-Aldrich) was dissolved in DMSO. A final concentration of 100 nM resveratrol was used for treatment of APP Gal4 SY5Y cells for a period of 10 hours. Following the treatments, the cells were washed twice with cold PBS and lysed with GLB (Promega, Inc.). Lysates were used for luciferase assay using Steady Glo (Promega, Inc.). Luciferase counts were normalized to total protein concentration that was measured using the BCA protein assay kit (Pierce, Inc). γ-secretase transition state inhibitor, L-685,458, was purchased from Sigma Aldrich and dissolved in DMSO.

Drosophila Methods: All flies were raised on standard cornmeal/molasses/agar media. For flies treated with L-685,458, drug was added to standard fly medium to a final concentration of 100 and 250 nM. For resveratrol treatment, drug was added to standard fly medium to a final concentration of 100nM. Since L-685,458 and resveratrol were initially dissolved in DMSO a vehicle only control (0.01% DMSO) was used (Greeve, Kretzschmar et al. 2004). Larvae were raised on L-685,458, resveratrol or vehicle containing food and scored for phenotypic effects once they eclosed (described below).

Drosophila genotypes used were $Sir2^{KG00871}$(Rogina and Helfand 2004), $Sir2^{05327a}$(Spradling, Stern et al. 1999), $Sir2^{2A-7-11}$(Furuyama, Banerjee et al. 2004), $Sir2^{EP2300}$(Rogina and Helfand 2004), *UAS:LacZ, UAS:GFP*, w^{1118} (Bloomington Stock Center, http://flybase.bio.indiana.edu/), *Canton S*, and *GMR-App-Gal4, UAS:Grim / Cyo*(Guo, Hong et al. 2003; Gross, Feldman et al. 2008). All crosses were performed at 25°C. *GMR-APP-Gal4, UAS:Grim*/ *Cyo* flies were outcrossed to both w^{1118} and *Canton S* genotypes to determine the reference eye phenotype for the *GMR-APP-Gal4, UAS:Grim* adult eyes, as previously described (Guo, Hong et al. 2003; Gross, Feldman et al. 2008). Adult eyes were immersed in 95% ethanol and photographed using a Canon PowerShot S70 digital camera mounted to a Leica Mz 12_5 stereomicroscope.

To study the effect of caloric restriction on *GMR-App-Gal4, UAS:Grim*/ *Cyo*(Guo, Hong et al. 2003; Gross, Feldman et al. 2008) flies, they were outcrossed to w^{1118} flies on either high-calorie (15% yeast, 15% sucrose, and 2% agar) or low-calorie (5% yeast, 5% sucrose, and 2% agar) food (Rogina and Helfand 2004) and the progeny were scored (+ to +++) for effects on eye phenotype. "+" is a rough eye, where at least one-half of the eye field is wild type. "++" is a rough eye where more than one-half of the

eye is rough, usually affecting the whole eye, but not always. "+++" is a strong rough eye where the entire eye is affected, and there is a strong loss of pigment in the posterior one-half of the eye. Fly lines expressing loss or gain of function mutations were crossed with AICD reporter flies (Guo, Hong et al. 2003; Gross, Feldman et al. 2008). Control flies are AD flies outcrossed to control for crossing to mutant lines.

Statistical Analysis: Values in the text and figures are presented as means ± standard errors of at least three independent experiments. Equal variance or separate variance two-sample student's t-test were used, as appropriate, to compare two groups. "*" indicates$p< 0.05$ and "**" indicates $p< 0.01$. For our *in vivo* studies, to determine if there is a statistically significant change in phenotype relative to wild type we performed a G-test of homogeneity and report the p-values. "n.s." indicates p-values greater than 0.05.

Results:

***In vitro caloric restriction modulates APP proteolysis*:** To initially examine the effect of *in vitro* caloric restriction on Aβ levels we treated HEK-293 cells transiently over-expressing APP_{695} with DMEM supplemented with 10% serum from rats that had either been fed *ad libitum* (AL) or had been calorically restricted (CR) (de Cabo, Furer-Galban et al. 2003). We observed that a 48 hour *in vitro* CR treatment resulted in a robust and significant decrease in secreted Aβ levels compared to *in vitro* AL treatment (Figure 2-1A). Specifically, there was an 82% decrease in secreted Aβ levels ($p=0.007$) in cells exposed to CR conditions compared to cells exposed to AL conditions. *In vitro* CR treatment also resulted in a significant, yet smaller decrease in intracellular, RIPA-soluble Aβ levels (15%, $p=0.04$) compared to the *in vitro* AL treatment (Figure 2-1B).

Our observation that *in vitro* CR decreased the levels of Aβ levels prompted us to determine if the AICD levels were similarly affected, since γ-secretase cleavage produces Aβ and AICD. To study the effect of *in vitro* caloric restriction on AICD production we used the AICD mediated transactivation assay utilizing an APP-Gal4 fusion protein and the Gal4-Luc reporter construct (Cao and Sudhof 2001). We utilized SH-SY5Y cells stably expressing the APP-Gal4 protein and carrying the Gal4-UAS driven firefly *Luciferase* reporter gene (Zhang, Khandelwal et al. 2007). The APP-Gal4 undergoes normal secretase-mediated processing to produce the AICD-Gal4 fragment. This fragment transactivates the Gal4-UAS responsive *Luciferase* reporter gene (Cao and Sudhof 2001). We have shown that changes in luciferase activity in this assay provide an accurate measure of changes in AICD-Gal4 levels (Zhang, Khandelwal et al. 2007). We exposed SY5Y-APP-Gal4 cells to *in vitro* AL or CR (utilizing F344 and FBN serum)

conditions for 48 hours. We observed that for the cells exposed to F344 CR conditions, there was a significant decrease (46%, $p<0.001$) in AICD-Gal4-mediated luciferase expression compared to AL conditions (Figure 2-1C). Similarly, in cells exposed to FBN CR conditions there was a 50% decrease in the luciferase expression compared to FBN AL treatment (Supplementary Figure 2-1) We also determined the steady state levels of AICD-Gal4 in these cells using Western blot analysis (Figure 2-1D). Consistent with the luciferase assay results, we observed that there was a decrease in the AICD-Gal4 protein levels in the cells exposed to CR (F344) conditions compared to the cells exposed to AL (F344) conditions. These findings suggest that *in vitro* CR exposure results in decreased levels of proteolytic products of APP by γ-secretase.

In vitro caloric restriction modulates full-length APP levels: To determine if these observed effects were due to decreases in secretase activity and/or substrate availability, we exposed SY5Y-APP-Gal4 cells to *in vitro* AL or CR conditions using serum from both F344 and FBN rats and determined steady state full-length APP-Gal4 levels. We observed a significant decrease (38%, $p<0.01$and 65% $p<0.05$ respectively) in total, full-length APP-Gal4 levels (mature and immature forms of APP-Gal4) in the cells exposed to CR conditions compared to the cells exposed to AL conditions (Figure 2-2A, 2-2B). We also exposed naïve SH-SY5Y cells to *in vitro* AL or CR (F344) conditions to determine the effect on endogenous levels of full-length APP (Figure 2-2C). Again, we observed a significant decrease (33%, $p<0.01$) in the endogenous APP levels in the cells exposed to CR conditions (Figure 2-2D). Our findings indicate that *in vitro* CR modulates the levels of full-length APP and its proteolytic fragments including Aβ. These findings suggest that *in vitro* CR exposure reduces Aβ levels in part by decreasing full-length APP

levels. Similar treatments in SY5Y-APP-Gal4 cells with media supplemented with FBN CR serum also led to a robust and significant decrease in full-length APP levels when compared to cells exposed to AL serum (Supplementary Figure 2-2A and 2-2B).

In vitro caloric restriction induces SirT1: In model organisms, caloric restriction extends life span by increasing the levels of Sir2 (Silencing Information Regulator 2) (Howitz, Bitterman et al. 2003; Denu 2005). *In vitro* CR conditions induce SIRT1 expression in HEK-293 cells and promotes increased resistance to apoptosis (Cohen, Miller et al. 2004). We exposed SY5Y-APP Gal4 cells and naïve HEK-293 cells to *in vitro* AL or CR conditions. We observed a robust increase in steady state levels of SirT1 in both SY5Y-APP Gal4 cells (Figure 2-2E, 2-2F) and HEK-293 cells (data not shown) when exposed to *in vitro* CR conditions compared to *in vitro* AL conditions.

SirT1 over-expression modulates APP metabolism: Next, we set out to determine if SirT1 expression was sufficient to recapitulate the effects of *in vitro* CR on APP metabolism. We transiently over-expressed SirT1 or a vector control in HEK-293 cells transiently over-expressing the Swedish variant of APP (APP Swed-293 cells). SirT1 over-expression led to a significant decrease (92%, $p=0.003$) (Figure 2-3A) in secreted $A\beta_{40}$ levels. To determine if AICD levels are also affected by SirT1 over-expression, we transiently over-expressed SirT1 or a vector control in SY5Y-APP-Gal4 cells along with a reporter plasmid *Renilla Luciferase* (to control for transfection efficiency). Transient over-expression of SirT1 led to a significant decrease (<80%, $p<0.01$) in AICD-Gal4-mediated firefly luciferase activity (Figure 2-3B).

To further determine if SirT1 over-expression mimics the effect of *in vitro* CR on the full-length APP steady state levels, we transiently and stably (data not shown) over-

expressed SirT1 or an empty vector control in SY5Y-APP-Gal4 cellscells. We observed that SirT1 over-expression does not affect full-length APP levels, compared to cells transfected with an empty vector (Figure 2-3C). SirT1 transient over-expression however led to an increase in α-secretase cleavage of APP leading to increased levels of sAPPα (Figure 2-3E).

To confirm that increased SirT1 deacetylase activity was responsible for the SirT1-mediated changes in APP metabolism, we exposed SY5Y-APP Gal4 cells to the SirT1 agonist resveratrol (100nM) or vehicle (DMSO) for 4 hours(Howitz, Bitterman et al. 2003). This treatment resulted in significantly decreased (60%, $p<0.01$) AICD-Gal4-mediated mediated luciferase activity (Figure 2-3D) and in significantly increased sAPPα levels (350%, $p=0.013$; Figure 2-3F,). This treatment did not affect full-length APP levels (data not shown). Thus, in our model, resveratrol treatment resulted in the same modulation of APP metabolism as we observed upon SirT1 over-expression but both of these did not result in the diminished full-length APP levels observed *uponin* vitroCR treatment

Caloric restriction modulates AICD-dependent rough-eye phenotype in reporter flies: To verify the effects of CR on APP metabolism in a model organism, we utilized the AICD reporter flies previously described for the developing *Drosophila melanogaster* eye (Guo, Hong et al. 2003; Gross, Feldman et al. 2008). This assay is based on a transgenic fly that expresses a C99-Gal4 fusion protein in the developing retina (Guo, Hong et al. 2003; Gross, Feldman et al. 2008). Endogenous secretase activity in the fly retina leads to the proteolytic cleavage of this fusion protein, ultimately releasing the AICD-Gal4 fragment from the membrane (Guo, Hong et al. 2003; Gross, Feldman et al.

2008) which activates the transcription of the *Drosophila* cell death activator *Grim*(Chen, Nordstrom et al. 1996). Expression of *Grim* causes apoptosis in retinal cells resulting in a rough eye phenotype with disrupted bristle morphology and loss of pigmentation (Figure 2-4A). To demonstrate that cell death in fly eyes were indeed APP metabolism dependent, we treated the reporter flies with a transition state inhibitor of γ-secretase, L-685,458, and/or vehicle (DMSO) and scored flies (+ to +++) for the effects on the *GMR-APP-Gal4, UAS:Grim* mediated rough eye phenotype. We observed that treatment with 100 nM or 250 nM L-685,458 significantly suppressed ($p<0.0001$) the rough eye phenotype (Table 2-1; Figure 2-4B compared to Figure 2-4A). Thus, alteration of APP metabolism in the reporter flies can lead to increased or decreased cell death resulting in enhancement or suppression of the rough eye phenotype which is readily observable. This *in vivo* APP metabolism reporter system is directly analogous to the approach we have utilized to measure AICD levels in cell culture.

To study the effect of caloric restriction on the AD phenotype, these AICD reporter flies (Guo, Hong et al. 2003; Gross, Feldman et al. 2008) were out-crossed to w-; +/+ flies on high calorie (HC) and low calorie (LC) food (Rogina and Helfand 2004) and the progeny were scored (+ to +++) for effects on eye phenotype. We observed that exposure to LC conditions significantly suppresses the rough eye phenotype of the AICD reporter flies (Figure 2-5 and Table 2-2).

Sir2 modulates AICD-dependent rough-eye phenotype in reporter flies: To verify the effects of SirT1 on APP metabolism in a model organism, we utilized the AICD reporter flies (Guo, Hong et al. 2003; Gross, Feldman et al. 2008). Fly lines expressing loss or gain of function mutations of the *Drosophila* SirT1 homologue Sir2 were crossed with

AICD reporter flies (Guo, Hong et al. 2003; Gross, Feldman et al. 2008) and the progeny were scored (+ to +++) for effects on eye phenotype (Figure 2-6 and Table 2-3A). We tested three independently generated *Sir2* loss-of-function alleles ($Sir2^{KG00871}$, $Sir2^{05327a}$,and $Sir2^{2A-7-11}$) for genetic modification of the *GMR-APP-Gal4*, *UAS:Grim*rough eye phenotype (Figure 2-6, Table 2-3A). In two of the three loss-of-function mutants, loss of *Sir2* significantly enhances the rough eye phenotype ($p<0.001$, Table 2-3A). This results in decreased eye size, enhanced pigmentation loss, and severely disrupted eye and bristle morphology (Figures 2-6B and 2-6C).

We then tested the effect of Sir2 gain-of-function on the rough eye phenotype. *Sir2* gain-of-function (*UAS:Sir2*) resulted in a significant dominant suppression of the rough eyephenotype ($p<0.02$, Figure 2-7, Table 2-3B). This suppression is particularly detectable in the anterior region (Figure 2-7B), where increased eye pigmentation and more normal eye and bristle morphology was observed. Given that this reporter fly phenotype relies on the ability of the cleaved APP-Gal4protein to bind to and activate the transcription of the *UAS:Grim*DNA sequence of retinal cells, insertion of another *UAS* DNA sequence (in this case *UAS:Sir2*) therefore might titrate away *APP-Gal4* binding sites, causing a suppression of the rough eye phenotype by decreasing the amount of Grim protein that is expressed. To rule out this possibility, we crossed *GMR-APP-Gal4*, *UAS:Grim*flies to both *UAS:LacZ*, and *UAS:GFP* chromosomes, as both proteins are innocuous when expressed in the developing eye (Al-Ramahi, Perez et al. 2007). In both cases, the rough eye phenotype was not altered (Figure 2-7A). This indicated that the suppression we observe due to *Sir2* gain-of-functionis specific to the expression of Sir2.

In our cell culture model we observed that the SirT1 agonist resveratrol recapitulated some of the effects of SirT1 over-expression on APP metabolism indicating the significance of SirT1 deacetylase activity for these changes. To test if Sir2 activity was important for suppressing the rough-eye phenotype in our fly model, we crossed the AICD reporter flies to the control (w^{-};+/+) flies on food containing 100 nM resveratrol and/or vehicle (DMSO) and scored the eyes of the F1 progeny for rough eye phenotype. Compared to vehicle (DMSO) we observed that resveratrol significantly suppressed the rough eye phenotype in the AICD reporter flies (Figure 2-4C and Table 2-1).

Discussion

Reducing the levels of Aβ is a major goal of AD therapeutics. Previous reports have demonstrated that CR diets reduced Aβ levels and neuropathology in different AD models (Patel, Gordon et al. 2005; Wang, Ho et al. 2005; Qin, Yang et al. 2006). In this study we show that *in vitro* CR reduces the levels of soluble and intracellular Aβ in HEK-293 cells. We also observed that *in vitro* CR led to a decrease in AICD levels and AICD mediated luciferase levels in SY5Y-APP-Gal4 cells. The effects of *in vitro* CR on Aβ and AICD levels seems to be in part due to a decrease in full-length APP levels.

Caloric restriction is a stressor and induces SIRT1 expression in a wide array of tissues and makes cells more resistant to stress (Brunet, Sweeney et al. 2004). SirT1 (or Sir2) expression alone has been shown to increase stress resistance and extend lifespan of model organisms (Kaeberlein, McVey et al. 1999; Tissenbaum and Guarente 2001; Rogina and Helfand 2004; Colman, Anderson et al. 2009). CR extends life-span by increasingthe activity of the NAD-dependent protein deacetylase SirT1(Hekimi and Guarente 2003). In our study, *in vitro* CR increases SirT1 levels as has been previously observed for this *in vitro* CR model (Cohen, Miller et al. 2004). We find that SirT1 over-expression or treatment with the SirT1 agonist, resveratrol, recapitulates some of the the*in vitro* CR mediated effects on APP metabolism. We specifically observed that over-expressing SirT1 led to a robust and significant decrease in secreted $A\square_{40}$ levels, AICD levels, and AICD-mediated transactivation. SirT1 over-expression mediated increases in sAPPα levels are consistent with an increase in α-secretase activity, as has been previously reported (Qin, Yang et al. 2006). Therefore, SirT1 mediated decreases in Aβ may be due to increased α-secretase activity.

We have previously observed that genetic and pharmacologic induction ofα-secretase activity results in increased AICD levels and increased AICD-mediated transactivation (Zhang, Khandelwal et al. 2007). Here, however, despite the SirT1-mediated induction of α-secretase activity we observe decreased AICD levels and transactivation. This suggests that in these cells SirT1 not only regulates APP metabolism by inducing α-secretase activity but also by negatively regulating full-length APP proteolysis possibly by inhibiting γ-secretase activity.

While the SirT1-mediated increase in α-secretase activity has been previously observed in primary mouse neurons (Qin, Yang et al. 2006), the *in vitro* CR mediated decreases in full-length APP levels that we observe have not been previously reported. This observation may be due simply to the cell types utilized here or it could be a true cellular response to CR. According to a very recent study, SirT1 appears to be a positive regulator of GH(Growth Hormone) production and signaling, and that SirT1 activity in the hypothalamus might decrease during CR (Cohen, Supinski et al. 2009). This indicates that there might be an indirect link between SirT1 and α-secretase activity through somatotropic signaling in the brain that is triggered by CR. It was also recently reported that SirT1 directly regulates autophagy by deacetylating Atg5, Atg7 and Atg8(Lee, Cao et al. 2008). Autophagy is a cellular response to limited nutrients to lysosomally degrade non-vital proteins and organelles to produce nutrients ensuring that vital cellular response can continue(Yorimitsu and Klionsky 2005). Lysosomal function(s) decline(s) in older animals. The decline is prevented by CR (Bergamini, Cavallini et al. 2003). In certain organs, like the brain, the proteasomal degradation pathway is more efficiently up regulated during long-term fasting. The proteasome acting together with ubiquitin and

ubiquitin-processing enzymes is responsible for most cytosolic protein degradation under normal nutrient conditions and has a variety of essential functions including protein quality control. It has been shown that during acute nutrient depletion, proteins can be degraded via the proteasomal pathway for generation of amino acids needed for cell maintenance (Takahashi and Goto 2002; Vabulas and Hartl 2005). We intend to investigate the role of both the

Finally, we wanted to determine if the effects of caloric restriction and SirT1 induction on APP processing that we observed in our *in vitro*model, are in fact recapitulated in an *in vivo* model system. In this model system, apoptosis in developing *Drosophila* eyes is dependent on AICD production as previously demonstrated using $PS1^{(-/-)}$ flies(Guo, Hong et al. 2003; Gross, Feldman et al. 2008). We further confirm this dependence on γ-secretase activity by treating these flies with a γ-secretase inhibitor (L-685,458) that suppressed the rough eye phenotype. We observed for the first time that caloric restriction or over-expression of Sir2 (the *Drosophila* homolog of SirT1) suppresses the rough-eye phenotype in the developing eye of AICD reporter flies. Alternatively, the rough eye phenotype was enhanced in reporter flies lacking a functional Sir2. We also observed that the Sir2 agonist resveratrol was able to mimic the effects of Sir2 gain-of-function by suppressing the rough eye phenotype in these flies. These *in vivo* studies strongly support our *in vitro* findings. *In vitro* SirT1 activation via over-expression or *in vitro* CR resulted in decreased Aβ and AICD levels which is analogous to the decreased GRIM expression and the resulting decreased rough-eye phenotype *in vivo*.

In summary, we have demonstrated that *in vitro* caloric restriction can modulate APP metabolism and that some of these effects seem to be mediated by SirT1. Our results demonstrate that in these cells SirT1 regulates APP metabolism by increasing α-secretase activity, decreasing APP and AICD levels, and possibly by decreasing γ-secretase activity. In fact it has been recently reported that levels of PS1, a component of γ-secretase, increases in the hippocampus of senescence-accelerated mouse model (SAMP8), indicating that aging indirectly leads to increased γ-secretase (Kumar, Franko et al. 2009). Since CR and SirT1 have both been reported to have anti-aging effect, our observations in this study might be due to an indirect effect on the levels of -secretase components and therefore γ-sevretase activity. We are extending our studies to further analyze these possibilities. Importantly we have demonstrated that in AICD reporter flies, caloric restriction and the *Drosophila* homologue of SirT1, Sir2, can independently regulate AICD-dependent rough-eye phenotype in a manner analogous to what we observe in cultured human cells. Our findings illuminate potentially novel mechanisms of APP metabolism regulation *in vitro* and *in vivo*.

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Figure Legends

Figure 2-1:*In vitro* caloric restriction modulates APP proteolysis. (A) Treatment of HEK-293 cells with CR serum-containing media for 48 hours resulted in a decrease in soluble Aβ levels (measured in the conditioned media) compared to treatment with AL serum containing media (p=0.007). (B) Treatment of HEK-293 cells with CR serum containing media for 48 hours resulted in a decrease in intracellular Aβ levels (measured in the cell lysates with the help of NP40) compared to treatment with AL serum containing media (p=0.04). Samples were in triplicates and error bars represent standard deviation. (C) Treatment of APPGal4 stably over-expressing SH-SY5Y (SY5Y-APP Gal4) cells with CR serum containing media for 48 hours resulted in a decrease in AICD mediated Firefly Luciferase expression compared to treatment with AL serum containing media. There were 12 samples per treatment and error bars represent standard error of the means. (D) CR serum treatment of SY5Y-APP Gal4 cells for 48 hours resulted in a decrease in the steady state levels of AICD-Gal4 compared to treatment with AL serum containing media. β-Actin was used as the loading control.

Figure 2-2:*In vitro* caloric restriction modulates full-length APP levels and induces SirT1. (A) CR serum treatment decreases full-length APP levels in SY5Y-APP Gal4 cells compared to treatment with AL serum containing media (48 hour treatment). β-Actin was used as the loading control. (B) Quantification of panel A blot shows a 38% decrease in the steady state levels of full-length APP in CR serum treated cells compared to AL serum treated cells. Treatments were done in triplicate and error bars represent standard error of the means. (C) Treatment of naive SH-SY5Y cells with CR serum containing

media for 48 hours resulted in a decrease in the steady state levels of full-length APP compared to treatment with AL serum containing media. β-Actin was used as the loading control. (D)Quantification of the blot shows a 33% decrease in the steady state levels of full-length APP in CR serum treated cells compared to AL serum treated cells. Treatments were done in triplicates and error bars represent standard error of the means. (E) Treatment with CR serum for 48 hours induces SirT1.Treatment with media containing CR serum increases the steady state levels of SirT1 compared to treatment with media containing AL serum in SY5Y-APP Gal4 cells. β-Actin was used as the loading control.

Figure 2-3: SirT1 over-expression modulates APP proteolysis. (A) Transient over-expression of SirT1 in APPSwed-293 cells decreased secreted $A\beta_{40}$ levels in comparison to cells transfected with the empty vector. There was ~92% ($p<0.01$) decrease in Aβ levels as a result of SirT1 over-expression. Sample number was 3 and error bars represent standard error of the means. (B) Transient over-expression of SirT1 in SY5Y-APP Gal4 cells decreased AICD mediated transactivation in comparison to cells transfected with the empty vector ($p<0.01$) where the Firefly Luciferase CPS were normalized to cell number. There was ~60% decrease in AICD mediated Firefly Luc expression as a result of SirT1 over-expression. Sample number was 4 and error bars represent standard error of the means. (C)Transient over-expression of SirT1 in SY5Y-APP-Gal4 cellsdoes not affect full-length APP levels in comparison to cells transfected with the empty vector. β-Actin was used as the loading control. (D) Transient over-expression of SirT1 in SY5Y-APP Gal4 also increased the levels of sAPPα (the soluble fragment of APP that is released due

to the α-secretase cleavage of full-length APP) compared to the cells expressing the empty vector. (E) SY5Y-APP Gal4 cells treated with 100 nM resveratrol for 6hours resulted in a decrease in AICD-mediated Luciferase expression when compared to vehicle (DMSO) alone ($p<0.01$).Treatments were done in triplicate, error bars represent standard error of the means. (F) SY5Y-APP Gal4 cells treated with 50 μM resveratrol for 4 hours results in increased levels of sAPPα compared to the cells treated with the vehicle (DMSO) control.

Figure 2-4: AICD-dependent rough-eye phenotype. All panels show adult *Drosophila* eyes from flies maintained at 25°C, same magnification. (A) Wild type eye. Note the regular array of ommatidia throughout the eye field. (B) *GMR-APP-Gal4*, *UAS:Grim*eye shows a smaller eye than wild type, with disrupted ommatidia and bristle morphology throughout the eye field. (C) *GMR-APP-Gal4*, *UAS:Grim*flies treated with vehicle (DMSO) . (D) *GMR-APP-Gal4*, *UAS:Grim* flies treated with γ-secretase inhibitor L-685,458 display a suppression of the rough eye phenotype.(E) *GMR-APP-Gal4*, *UAS:Grim* flies treated with Sir2 agonist resveratrol display a suppression of the rough eye phenotype.

Figure 2-5: Caloric restriction modulates AICD-dependent rough-eye phenotype. All panels show adult *Drosophila* eyes from flies maintained at 25°C, same magnification. (A) *GMR-APP-Gal4, UAS:Grim*/ *w*- flies maintained in high calorie foodshow no significant modification of the rough eye phenotype as compared to *GMR-APP-Gal4*,

UAS:Grim. (B) *GMR-APP-Gal4, UAS:Grim*/ *w-* flies maintained in low calorie foodshows a partial rescue of the *GMR-APP-Gal4, UAS:Grim* rough eye phenotype.

Figure 2-6: Loss-of-function mutations in the *Sir2* gene modulate AICD-dependent rough-eye phenotype. All panels show adult *Drosophila* eyes from flies maintained at 25°C, same magnification. (A) *GMR-APP-Gal4, UAS:Grim*eye shows a smaller eye than wild type, with disrupted ommatidia and bristle morphology throughout the eye field. (B) *GMR-APP-Gal4, UAS:Grim*/ $Sir2^{KG00871}$shows a smaller eye than *GMR-APP-Gal4, UAS:Grim*, with increased loss of pigmentation (arrow). (C) *GMR-APP-Gal4, UAS:Grim*/ $Sir2^{05327a}$shows a smaller eye, with increased loss of pigmentation (arrow) than *GMR-APP-Gal4, UAS:Grim*.

Figure 2-7: Gain-of-function mutations in *Sir2* gene modulate AICD-dependent rough-eye phenotype. All panels show adult *Drosophila* eyes from flies maintained at 25°C, same magnification. (A) *GMR-APP-Gal4, UAS:Grim*/ *UAS: LacZ* shows no significant modification of the rough eye phenotype as compared to *GMR-APP-Gal4, UAS:Grim*. (B) *GMR-APP-Gal4, UAS:Grim*/ *UAS:Sir2* (*Sir2*^{EP2300}allele) shows a partial rescue of the *GMR-APP-Gal4, UAS:Grim* rough eye phenotype, particularly in the anterior region (arrow).

Table.2-1: Quantitative analysis of γ-secretase inhibition as a result of L-685,458 treatment. AICD reporter flies (Guo, Hong et al. 2003; Gross, Feldman et al. 2008) were fed L-685,458, the γ–secretase inhibitor and the progeny were scored (+ to +++) for

effects on eye phenotype. “+” is a rough eye, where at least ½ of the eye field is wild type. “++” is a rough eye where more than ½ of the eye is rough, usually affecting the whole eye, but not always. “+++” is a strong rough eye where the entire eye is affected, and there is a strong loss of pigment in the posterior ½ of the eye. To determine if there is a statistically significant change in phenotype relative to vehicle (DMSO) treated flies, we performed a G-test and report the p-values. “n.s.” indicates p-values greater than 0.05. Control flies are AD flies untreated or treated with 0.01 %DMSO.

Table.2-2: Quantitative analysis of pathological alteration in AD flies as a result of caloric restriction. AD flies(Guo, Hong et al. 2003; Gross, Feldman et al. 2008)were out-crossed to w- flies on high and low calorie food (Rogina and Helfand 2004)and the progeny were scored (+ to +++) for effects on eye phenotype. To determine if there is a statistically significant change in phenotype relative to wild type we performed a G-test and report the p-values.

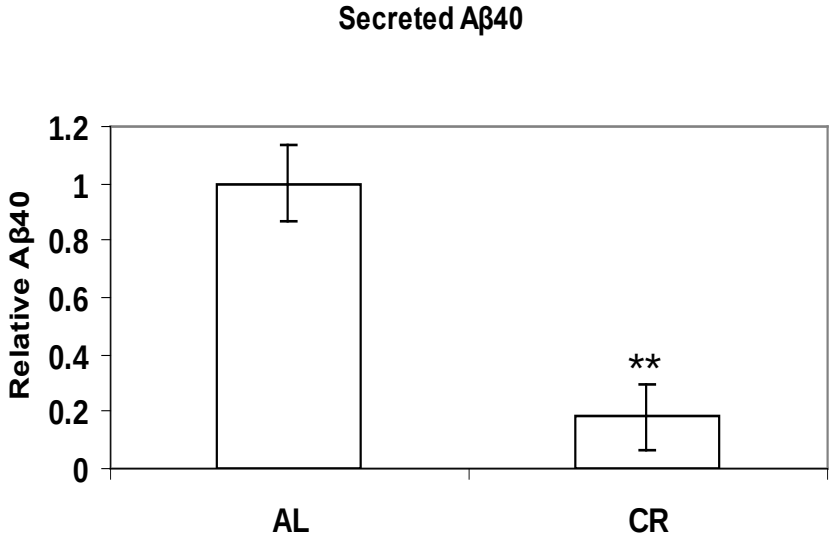
Table.2-3: Quantitative analysis of pathological alteration in Sir2 loss-of-function and gain-of-function mutant flies. Fly lines expressing Sir2 loss or gain-of-function mutations were crossed with AD flies(Guo, Hong et al. 2003; Gross, Feldman et al. 2008) and the progeny were scored (+ to +++) for effects on eye phenotype. To determine if there is a statistically significant change in phenotype relative to wild type we performed a G-test and report the p-values. “n.s.” indicates p-values greater than 0.05. *Control flies are AD flies out-crossed to control for crossing to mutant lines. (A) Sir2 loss-of-function mutant flies *GMR-APP-Gal4, UAS:Grim/ Sir2*^{KG00871} *and GMR-APP-Gal4, UAS:Grim /*

Sir2^{05327a} showed a significant (<0.0001) change in eye morphology and loss of pigmentation compared to the wild type control flies. (B) The Sir2 gain-of-function mutant fly line *GMR-APP-Gal4, UAS:Grim*/ *UAS:Sir2* (*Sir2*^{EP2300}allele) shows a significant ($p<0.02$) rescue of the rough eye phenotype.

Figures

Figure 2-1. *In vitro* CR decreases $A\beta_{40}$ levels

A



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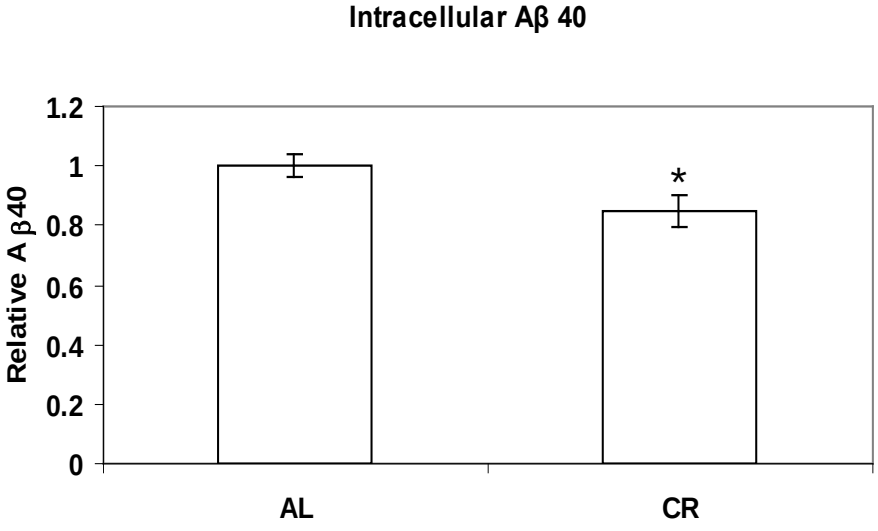


Figure 2-1C. *In vitro* CR decreases AICD mediated luciferase activity

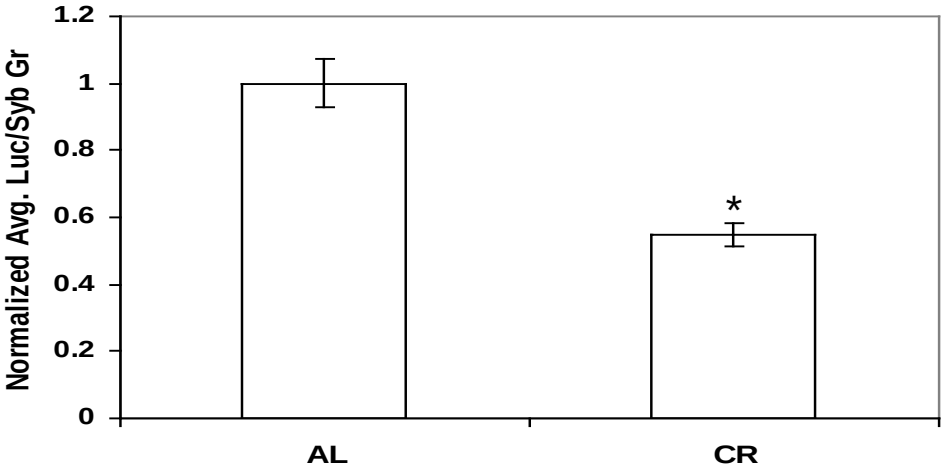


Figure 2-1D. *In vitro* CR decreases AICD levels

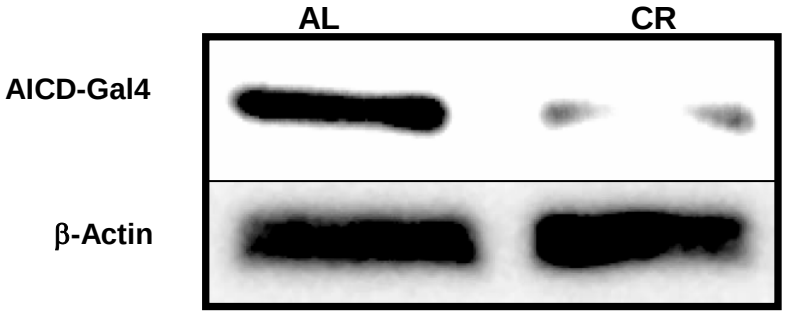


Figure 2-2A.*In vitro* CR decreases full-length APP levels in SY5Y-APP-Gal4 cells

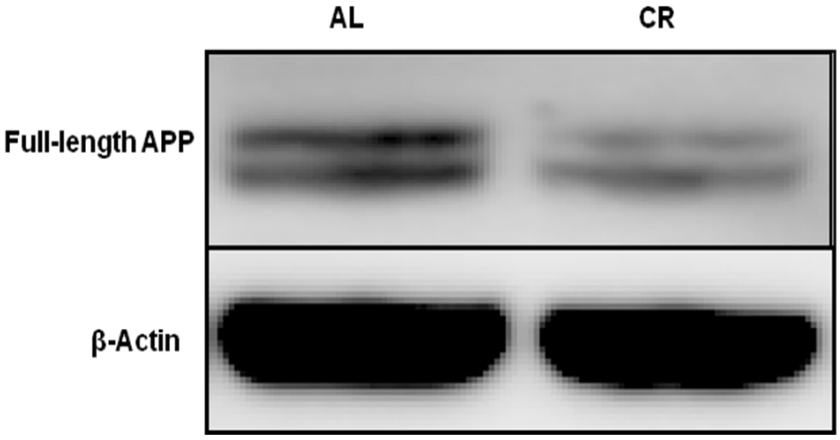


Figure 2-2B. Quantification of above Western Blot

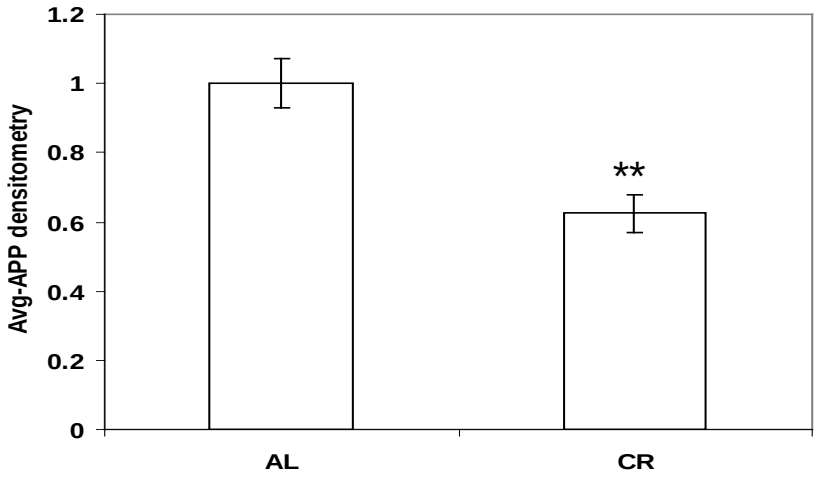


Figure 2-2C.*In vitro* CR decreases full-length APP levels in naïve SY5Y cells

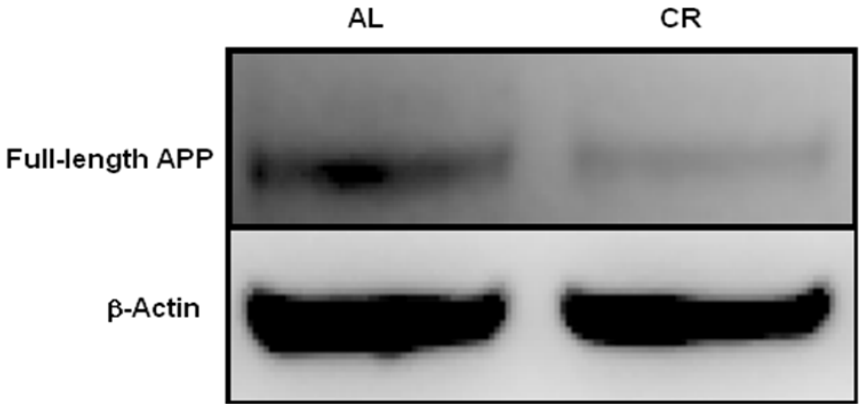


Figure 2-2D. Quantification of above Western Blot

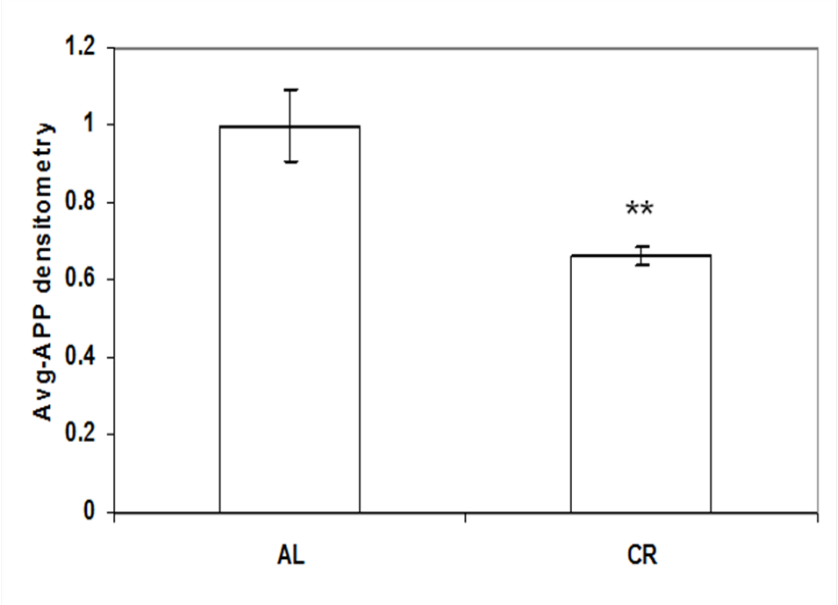


Figure 2-2E.*In vitro* CR decreases increases SirT1 levels in naïve SY5Y-APP-Gal4 cells

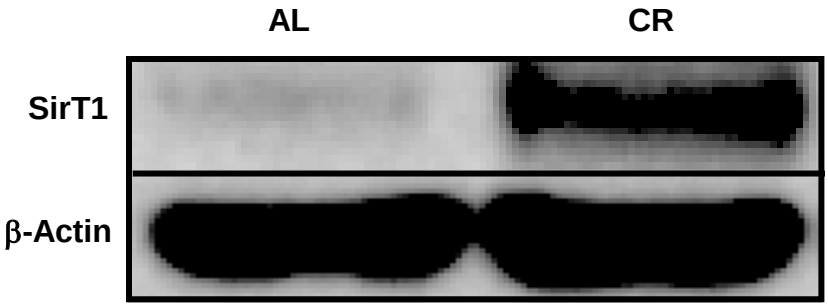


Figure 2-2F. Quantification of above Western Blot

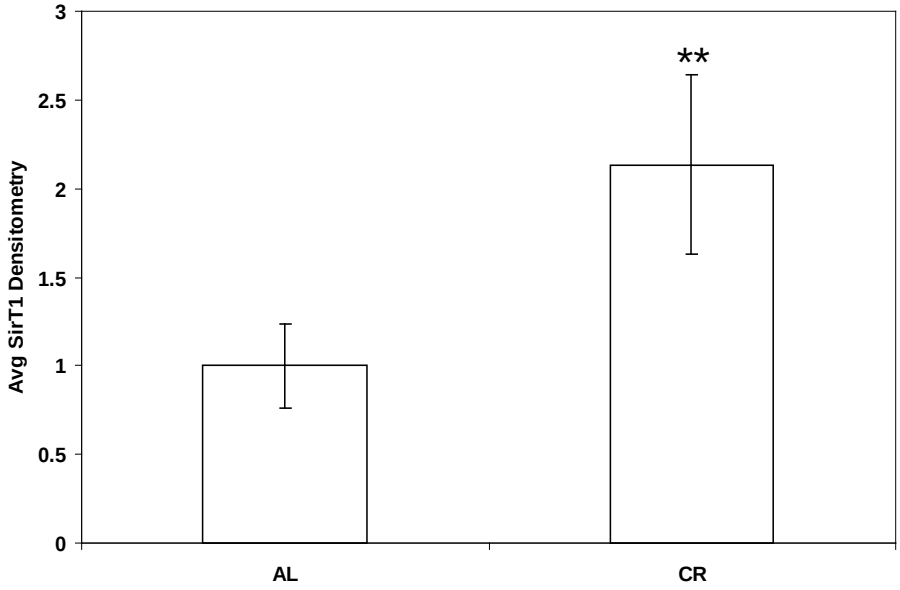


Figure 2-3A. SirT1 over-expression decreases $A\beta_{40}$ levels

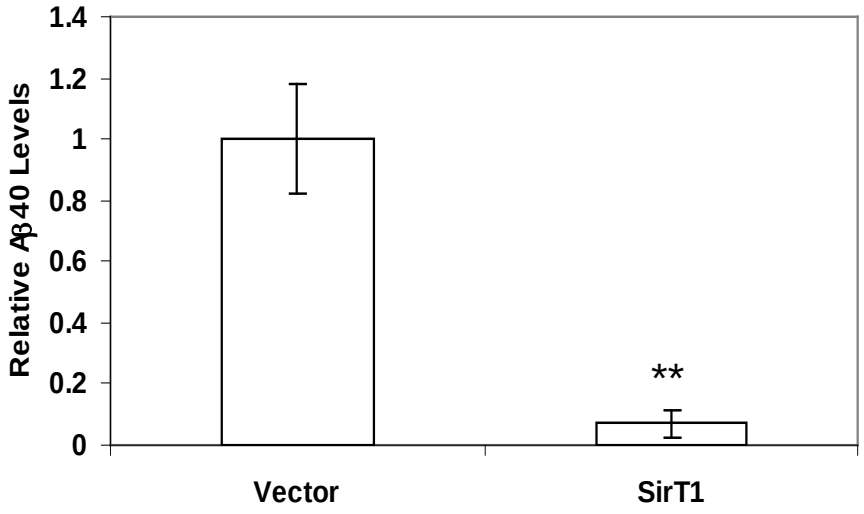


Figure 2-3B. SirT1 over-expression decreases AICD mediated luciferase levels

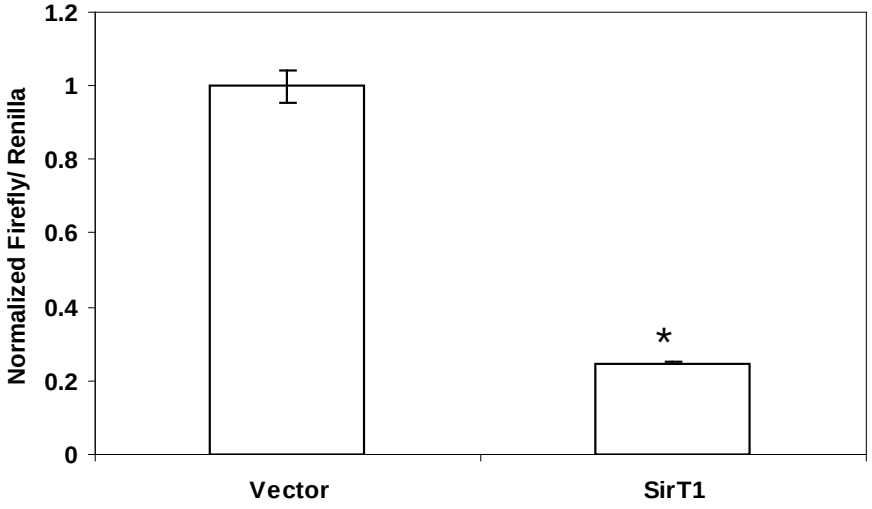


Figure 2-3C. SirT1 does not affect full-length APP levels

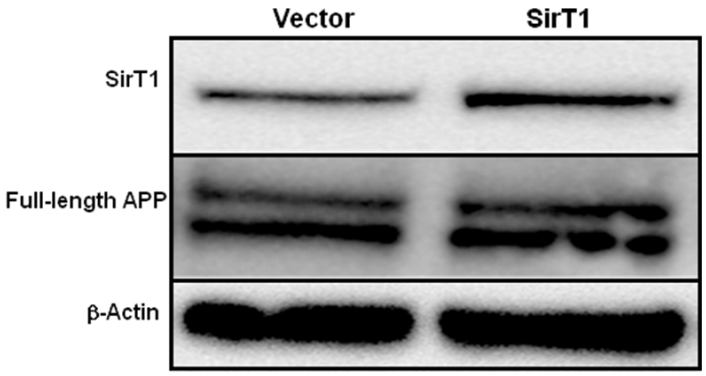


Figure 2-3D. SirT1 increases sAPPα levels

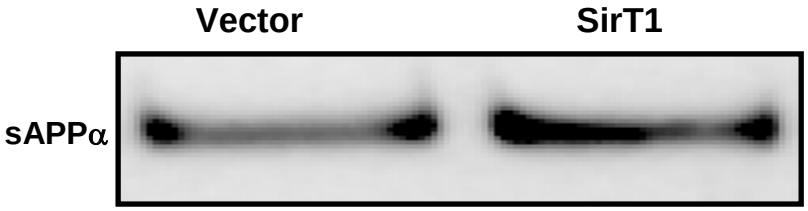


Figure 2-3D. Resveratrol treatment decreases AICD mediated luciferase levels

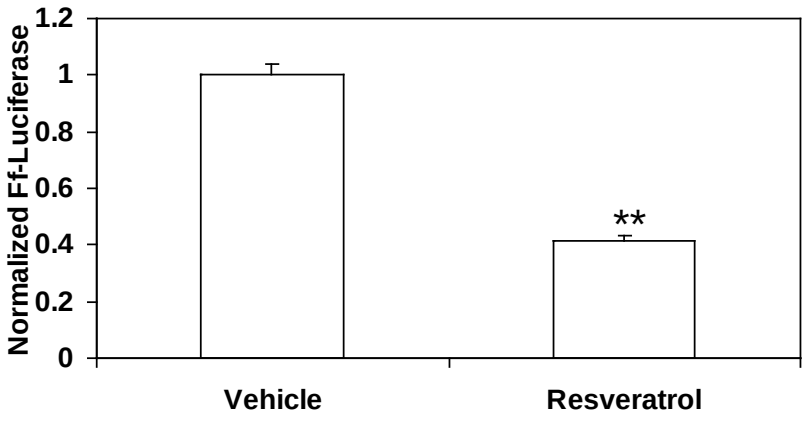


Figure 2-3E. Resveratrol treatment increases sAPPα levels

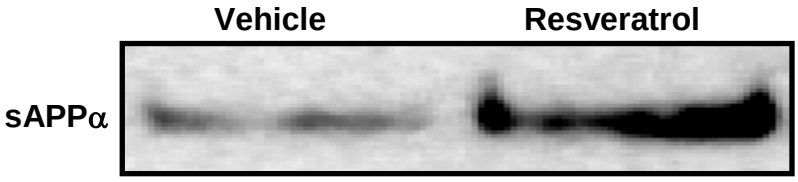
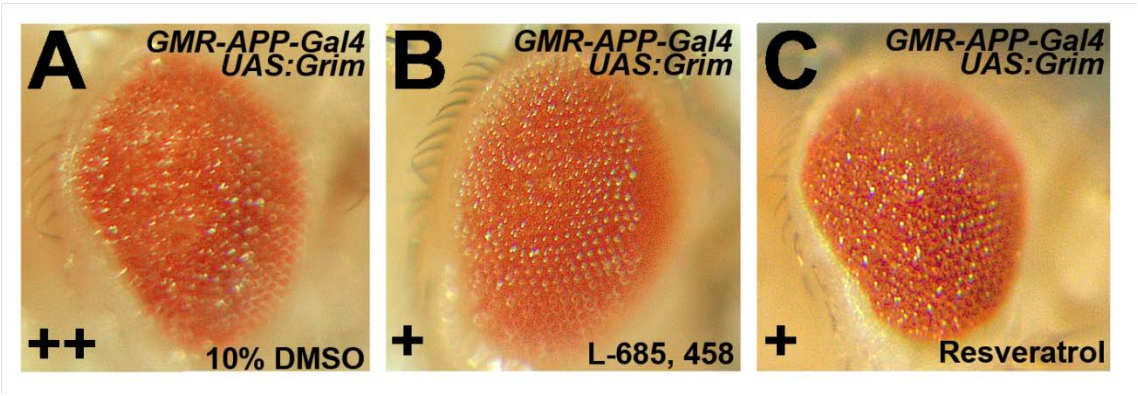


Figure 2-4. L-685,458 and Reaveratrol rescues rough-eye phenotype in AD flies



D

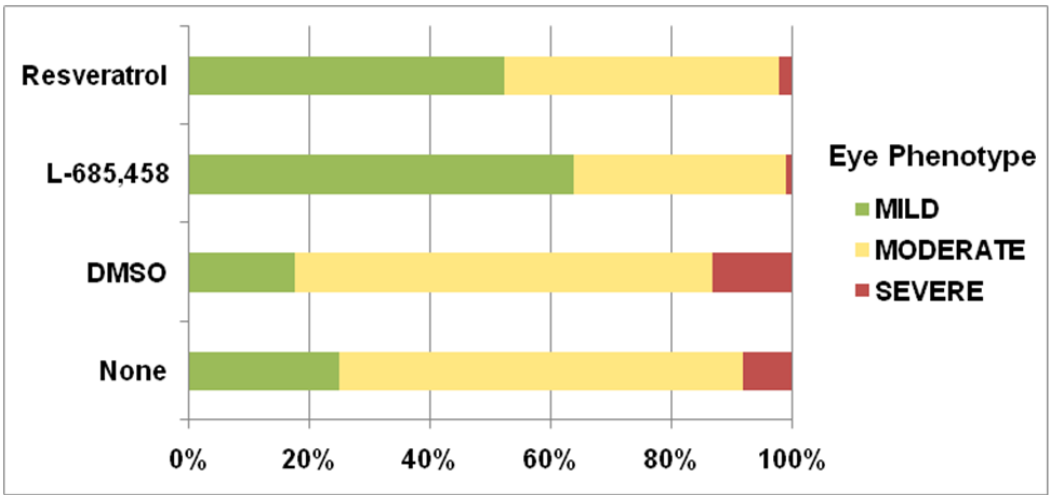
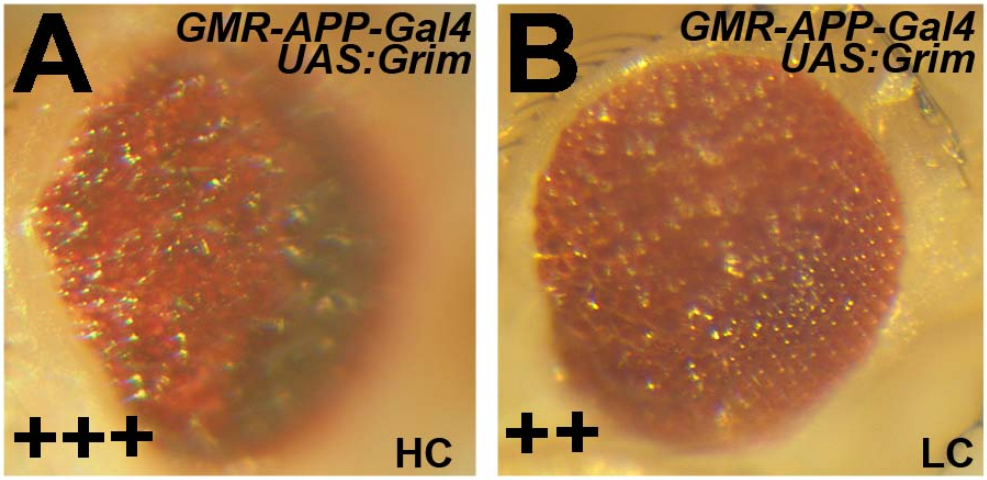


Figure 2-5. Caloric restriction rescues rough-eye phenotype in AD flies



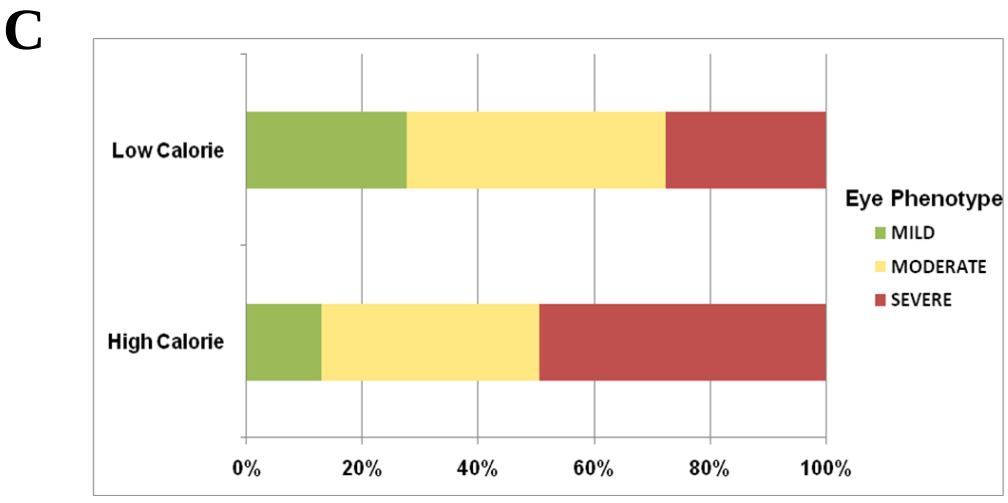
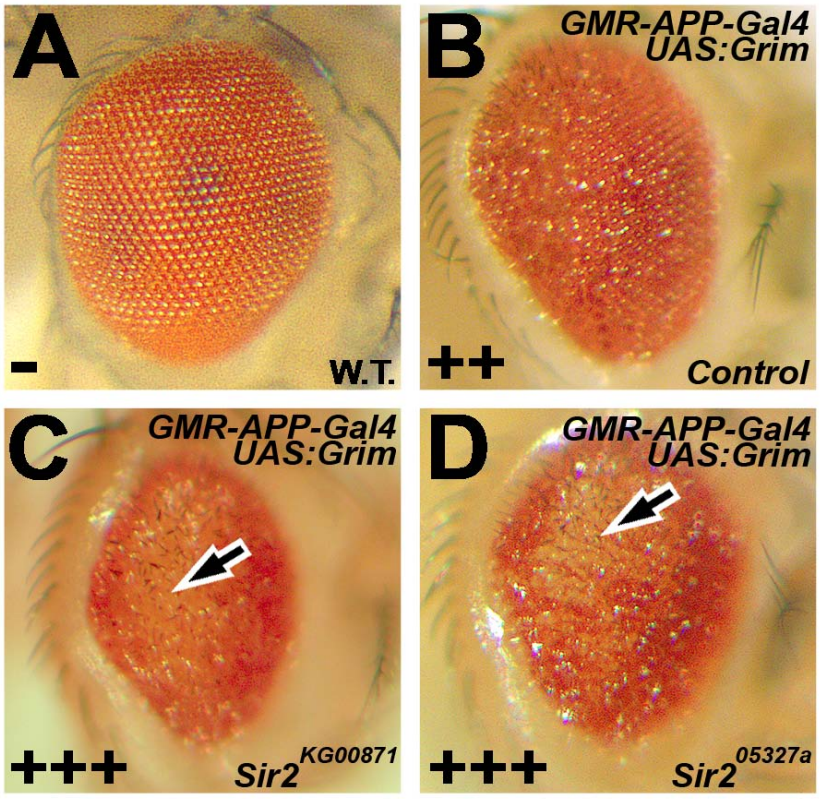


Figure 2-6. Sir2 loss-of function enhances rough-eye phenotype in AD flies


A
-
W.T.
B
GMR-APP-Gal4
UAS:Grim
++
Control
C
GMR-APP-Gal4
UAS:Grim
+++
Sir2KG00871
D
GMR-APP-Gal4
UAS:Grim
+++
Sir205327a

E

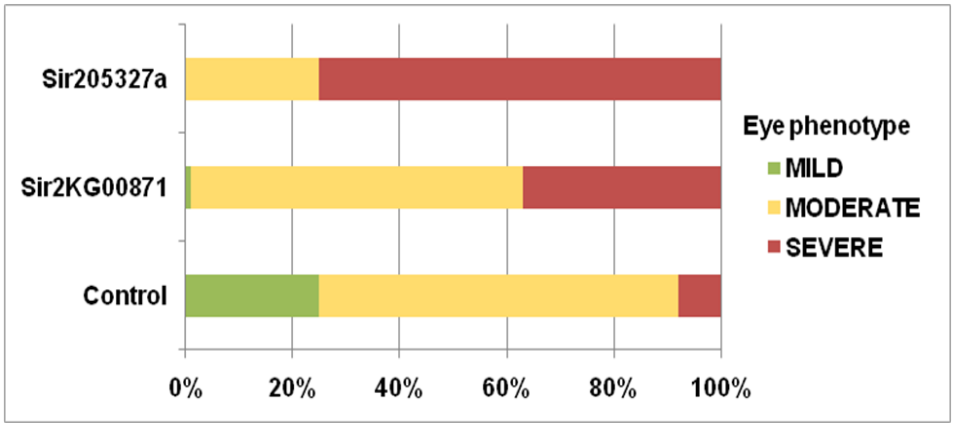
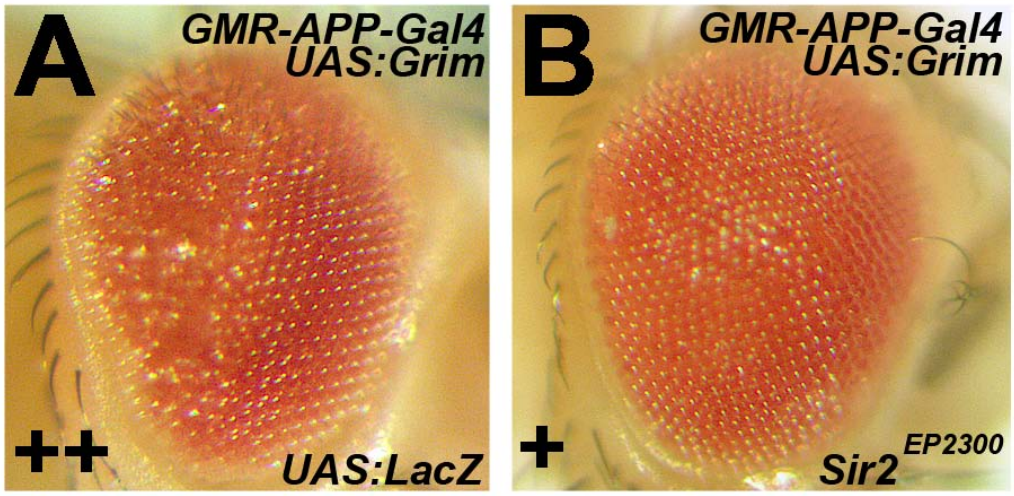

Sir205327a
Sir2KG00871
Control
0%
20%
40%
60%
80%
100%
Eye phenotype
MILD
MODERATE
SEVERE

Figure 2-7. Sir2 gain-of function rescues rough-eye phenotype in AD flies



C

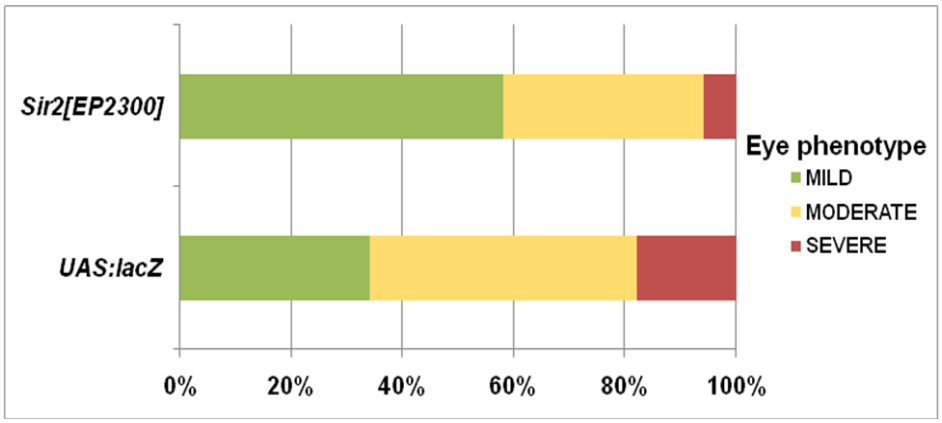


Table.2-1. Quantitative analysis of APP metabolism inhibition as a result of L-685,458 and resveratrol treatment

			% showing phenotype				
Drugs	**Dose**	**n**	+	++	+++	**p-value***	**Nature of Drug**
None	-	64	25	67	8		-
DMSO	-	87	23	90	17	n.s	Vehicle
L-685,458	100 nM	65	64	35	1	<0.0001	γ-secretase inhibitor
L-685,458	250 nM	95	53	44	3	<0.0001	γ-secretase inhibitor
Resveratrol	100 nM	84	52	45	2	<0.002	Sir2 Agonist

Table.2-2. Diet modulates APP metabolism in Drosophila model of AD

			% showing phenotype				
Type of Food	**Genotype**	**n**	+	++	+++	**p-value**	**Nature of allele**
Normal	w^-	64	25	67	8	-	Out-crossed to wildtype
High Calorie	w^-	157	13	38	50	<0.001	Out-crossed to wildtype
Low Calorie	w^-	130	28	45	28	<0.001	Out-crossed to wildtype

Table.2-3. Modulation of APP Metabolism in a Drosophila model of AD

A

			% showing phenotype				
Genes	**Genotype**	**n**	+	++	+++	**p-value**	**Nature of allele**
Control	w^{-}	137	37	55	8	-	Out-crossed to wildtype
Sir2	$Sir2^{KG00871}$	77	1	62	37	<0.0001	Loss-of-function
Sir2	$Sir2^{2A-7-11}$	95	30	62	8	n.s	Loss-of-function
Sir2	$Sir2^{05327a}$	72	-	25	75	<0.0001	Loss-of-function

B

			% showing phenotype				
Genes	**Genotype**	**n**	+	++	+++	**p-value**	**Nature of allele**
Control	UAS:lacZ	129	34	48	18	-	Out-crossed to UAS:lacZ
Sir2	$Sir2^{EP2300}$	97	58	36	6	<0.02	Gain-of-function

Supplementary Figures

Figure 2-1A. *In vitro* CR using serum from FBN rats decreases AICD mediated luciferase activity

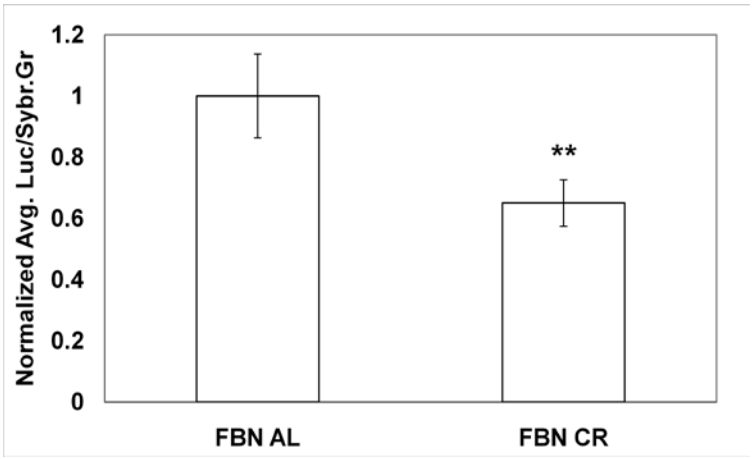


Figure 2-1B.*In vitro* CR using serum from FBN rats decreases full-length APP levels

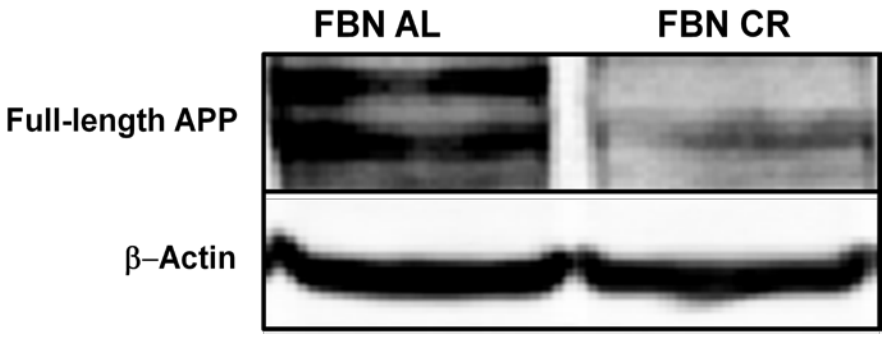
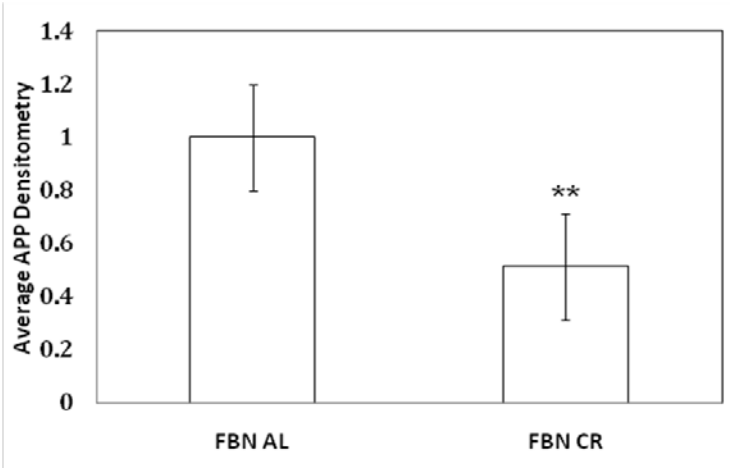


Figure 2-1C. Quantification ofabove Western Blot



CHAPTER 3

NUTRIENT AVAILABILITY MODULATES APP METABOLISM

Introduction

It is known that diet strongly influences the incidence and outcome in major age-related diseases including diabetes, obesity and vascular disease (Weindruch 1996). This opens an avenue of exploration in understanding how the factors that can regulate aging related processes might also regulate APP(Amyloid Precursor Protein) processing, deposition of β- amyloid peptide (Aβ) and therefore formation of amyloid plaques which is the hallmark of AD pathology(Hardy and Selkoe 2002; Tanzi and Bertram 2005). Excessive accumulation of Aβ could be due to an increase in its overall expression/production or due to a decrease in the degradation of the peptide (Caccamo, Oddo et al. 2005). Recent findings have also extended the influences of caloric restriction on AD (Wang, Ho et al. 2005; Qin, Chachich et al. 2006; Halagappa, Guo et al. 2007).

It has been widely known that excess consumption of sugar plays an important role in the epidemic of obesity around the world(Bray, Nielsen et al. 2004). A large body of epidemiological studies has suggested that type-2 diabetes mellitus is associated with an increased risk of AD (reviewed in (Cao, Lu et al. 2007)). It has also been shown that consumption of sucrose-sweetened water induced insulin resistance and exacerbated AD-like memory impairment and cerebral amyloidosis in APP/PS1 double transgenic mouse model of AD (Cao, Lu et al. 2007). A direct link has been established between diabetes and AD (Xu, von Strauss et al. 2009). However the mechanism by which type-2 diabetes mellitus may affect AD, are not well understood. It has been previously shown that insulin significantly reduces intracellular accumulation of Aβ by accelerating it's

trafficking from the trans-Golgi network to the plasma membrane. It has been further shown that insulin increases the extracellular level of Aβ by reducing its degradation via IDE (Insulin Degrading Enzyme) (Gasparini, Gouras et al. 2001). Thusseveral lines of evidence indicate that decreases in insulin levels and metabolic abnormalities pertinent to diabetes may affect the generation and degradation of Aβ {reviewed in (Craft and Watson 2004; Neumann, Rojo et al. 2008)}.In fact, study of components of serum (obtained from calorically restricted F344 rats), utilized in an *in vitro* model of caloric restriction showed a decrease in glucose levels and increase in insulin and IGF1 levels (Figure. 3-1). This model recapitulated some of the beneficial effects of CR on animal models like increased resistance to heat sock and oxidative shock.

In the present study we utilized an *in vitro* model and determined the effect of individual nutrients like glucose and pyruvate on APP metabolism. We show here that exposure to high glucose and pyruvate levels increases AICD mediated transactivation and full-length APP levels.

Materials and Methods

Cell culture and Treatments: SH-SY5Y cells (obtained from ATCC) were cultured in Dulbecco's modified Eagles medium, supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml) and streptomycin (25,000 υg/ml). SH-SY5Y cells stably expressing APP Gal4 and Gal4 UAS reporter construct (SY5Y-APP Gal4 cells) and HEK-293 cells stably transfected with Swedish APP_{695} plasmid (APPSwed-293 cells) were cultured in Dulbecco's modified Eagles medium, supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml),streptomycin (25,000 μg/ml) and G418 (200 μg/ml). For Glucose and Pyruvate treatments SY5Y-APP Gal4 cells were exposed for 6 hours to different concentrations of glucose and pyruvatic acid dissolved in PBS with 0.5% BSA.

Plasmids: For the APP transactivation assay, we used pMSt APP (APP Gal4) construct and pG5e1B-luc (Gal4 reporter plasmid). Both the plasmids were a generous gift from Cao and Sudhof (2001). pMst is a Gal4 expression vector driven by the SV40 promoter derived from pM (Clontech) by mutating the stop codon before the Gal4 DNA binding domain. pMst-APP encodes APP-Gal4. It was generated by cloning a PCR fragment containing the extracellular and transmembrane region of human APP_{695} (APPe, residues 1 to 651) into the Nhe I site of pMst-APPct (linker sequence between APPe and Gal4 = MLKK*PLASSR*MKLLS) (Cao and Sudhof 2001). pG5E1B-luc is the Gal4 reporter plasmid in which luciferase mRNA is driven by five copies of Gal4 UAS. pcDNA3.1, (G418 resistant plasmid, (Invitrogen)) was co-transfected. Renilla luciferase plasmid pRLSV40 was obtained from Promega(Zhang, Khandelwal et al. 2007).

APP transactivation assay: For the transactivation assay, SH-SY5Y cells were stably transfected with pMSt APP (APP Gal4) construct and pG5e1B-luc (Gal4 reporter plasmid) and the neomycin (G418) resistant plasmid pcDNA3.1 (Invitrogen). The cells were maintained in Dulbecco's modified Eagles medium, supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml), streptomycin (25,000 ug/ml) and G418 (200 ug/ml). Following glucose and/or pyruvate treatments in 24/96 well plates, the cells were washed twice with cold PBS and then lysed with GLB (GloLysis Buffer, Promega Inc.). Then Luciferase activity was measured using Steady Glo luciferase reagent, Promega Inc. The luciferase activity was measured using the TopCount plate reader (PerkinElmer.Inc). The luciferase counts per second (CPS) were normalized to cell count by Sybergreen Assay. For Syber green assay, 20x Syber Green (diluted with PBS from 1000x stock) was used. Experiments were done in triplicates. For the Dual luciferase assay, Renilla luciferase plasmid pRLSV40 (Promega) is transiently transfected in SH-SY5Y cells stably over-expressing pMSt APP (APP Gal4) construct and pG5e1B-luc (Gal4 reporter plasmid)**(Figure 3-1)**. For the Dual Luciferase assay cells were washed twice with cold PBS and lysed with Dulbecco's modified Eagles medium, supplemented with 10% fetal bovine serum, penicillin (25,000 U/ml),streptomycin (25,000 ug/ml) and G418 (200 ug/ml). Measurement of firefly luciferase activity (dependent on APP processing) and Renilla luciferase activity (independent of APP processing) are done using the Dual Glo kit (Promega). In the case of the Dual Luciferase Assay, the firefly luciferase counts were normalized to Renilla luciferase counts.

Western Blot Analysis: After the treatments, cells were washed twice with cold PBS and then lysed in RIPA Buffer containing different protease inhibitors [Antipain(100uM),

Aprotinin (2 ug/ml), Benzamide (15 ug/ml), Chymostatin (100 uM), Leupeptin (100 uM), Pepstatin A (1uM), PMSF (1uM), Sodium Metabisulfite(0.1 nM). 10ul of lysates were used for protein assay with the help of BCA Protein Assay Kit (Pierce, Inc.). The BCA protein utilizes a standard curve generated by RIPA standards (different dilutions of BSA protein in RIPA buffer). According to the protein concentrations, samples for Western Blot were prepared using the 4x Nupage LDS sample buffer (Invitrogen, Inc.) containing 0.2% BME (β-Merkaptoethanol, Sigma Aldrich). Equal volumes of protein were loaded on to each well of NuPAGE 4-12% BisTris Gel. From the gel the proteins were transferred on to 0.25 μ PVDF membrane (Millipore) using a semi-dry transfer apparatus. Blots were probed with different antibodies and the target protein densitometry was normalized to actin densitometry using a Flurochem 8900 gel imaging system (Alpha Innotech).

Antibodies used: APP C-terminal antibody (A8717; Sigma Aldrich, Inc), monoclonal anti β-Actin (A5441, Sigma Aldrich, Inc), monoclonal anti SirT1 (05-707, Upstate, Inc), APP 6E10 (ab10146, Abcam), goat anti-rabbit (NA934V, GE Healthcare UK Limited) and goat anti-mouse (NA931V, GE Healthcare UK Limited).

Statistical Analysis: Values in the text and figures are presented as means ± standard deviation of at least three independent experiments. Equal variance or separate variance two-sample student's t-test were used, as appropriate, to compare two groups. "*" indicates$p < 0.05$ and "**" indicates $p < 0.01$.

Results

Glucose and Pyruvate modulate APP proteolysis: To determine the effect of glucose on AICD production we used the AICD mediated transactivation assay utilizing an APP-Gal4 fusion protein and the Gal4-Luc reporter construct (Cao and Sudhof 2001). We utilized SH-SY5Y cells stably expressing the APP-Gal4 protein and carrying the Gal4-UAS driven firefly *Luciferase* reporter gene (Zhang, Khandelwal et al. 2007). The APP-Gal4 undergoes normal secretase-mediated processing to produce the AICD-Gal4 fragment. This fragment transactivates the Gal4-UAS responsive *Luciferase* reporter gene (Cao and Sudhof 2001). We have shown that changes in luciferase activity in this assay provide an accurate measure of changes in AICD-Gal4 levels (Zhang, Khandelwal et al. 2007). We exposed SY5Y-APP-Gal4 cells to different concentration of glucose and/or pyruvatedissolved in PBS with 0.5% BSA (Bovine Serum Albumin). We observed that there was a decrease in AICD-Gal4 mediated luciferase expression in these cells with decreasing glucose **(Figure 3-2A)**and/ or Pyruvate**(Figure 3-2B)**concentrations. We wanted to determine if the effect of decreasing levels of glucose and pyruvate on AICD mediated transactivation was due to general transcriptional repression. So we exposed SY5Y-APP-Gal4 cells transiently expressing pRLSV40 (Renilla Luciferase plasmid, whose expression was independent of APP processing) to different concentrations of glucose and pyruvate. We observed that there was still a significant decrease in AICD mediated transactivation in decreased level of glucose or pyruvate**(Figure 3-2C)**, suggesting that APP proteolysis is specifically affected in these conditions.

Glucose and Pyruvatemodulate full-length APP levels: To determine if these observed effects were due to decreases in secretase activity and/or substrate availability, we

exposed SY5Y-APP-Gal4 cells to different concentrations of glucose and determined steady state full-length APP-Gal4 levels. We observed a decrease in total, full-length APP-Gal4 levels (mature and immature forms of APP-Gal4) in the cells with decreasing concentrations of glucose **(Figure 3-2Aand 2B)**. We also exposed naïve SH-SY5Y cells to differentconcentrationsofglucoseto determine the effect on endogenous levels of full-length APP.Again, we observed a decrease in the endogenous APP levels with decreasing concentrations of glucose**(Figure 3-2C and 2D)**. These findings suggest glucose effects full-length APP levels. Similarly we also observed in SY5Y-APP-Gal4 cells that decreasing concentrations of pyruvatedecrease full-length APP levels **(Figure 3-2E and 3F)**.

Discussion

Current therapeutics available for AD treats the symptoms, but do not affect the underlying causes of the disease. Development of possible therapy that would target APP proteolysis and thereby Aβ generation would be extremely beneficial. A number of studies have shown that caloric restriction reduced Aβ levels and neuropathology in a variety ofAD models (Patel, Gordon et al. 2005; Wang, Ho et al. 2005; Qin, Yang et al. 2006). It has been shown that glucose and insulin levels specifically affect AD pathology (Gasparini, Gouras et al. 2001; Craft and Watson 2004; Cao, Lu et al. 2007). It was also reported in an *in vitro* model that there is a decrease in glucose levels with caloric restriction (de Cabo, Furer-Galban et al. 2003). Here we show that both glucose and pyruvate modulate APP metabolism.

We specifically observed that decreasing concentrations of glucose and pyruvate decrease AICD mediated luciferase levels in APP-Gal4 over-expressing SH-SY5Y cells. However,we wanted to determine if the effect of decreasing glucose and pyruvate levels that we observed was due to a general transcriptional repression. We observed that in SY5Y-APP-Gal4 cells AICD mediated transactivation was specifically affected due to changes in glucose and pyruvate levels. We also observed that decreased glucose levels also decreased C99-Gal4 levels in SY5Y-APP-Gal4 cells. This suggests that glucose levels might also affect β-secretase cleavage of APP and therefore decreases the amount of substrate available for γ-secreatse cleavage, leading to a decrease in AICD levels. We further observed that decreasing levels of glucose and Pyruvate decreased steady-state full-length APP levels. Therefore the effect of availability of these nutrients on AICD mediated transactivation, seem to be in part due to reduced levels of full-length APP. In

future we would determine the effect of availability of these specific nutrients affect α–, β– and γ–secetase activities and their products specifically Aβ levels. We would also extend out studies to determine if the effects observed on full-length APP are transcriptional or due to increased turnover.

In certain organs, like the brain, the proteasomal degradation pathway is more efficiently upregulated during long-term fasting. The proteasome acting together with ubiquitin and ubiquitin-processing enzymes is responsible for most cytosolic protein degradation under normal nutrient conditions and has a variety of essential functions including protein quality control. It has been shown that during acute nutrient depletion, proteins can be degraded via the proteasomal pathway for generation of amino acids needed for cell maintenance (Takahashi and Goto 2002; Vabulas and Hartl 2005). Reduction of glucose levels is similar to fasting in his in vitro model. We want to extend our studies to determine if the effect of nutrient availability on APP metabolism in our model is dependent on proteasomal and lysosomal activity. We would also monitor the levels of Notch, another typeI transmembrane protein similar to APP to determine if the effects of glucose and pyruvate are general and not specifically on APP.

Figures

Figure 3-1. Model for dual luciferase assay

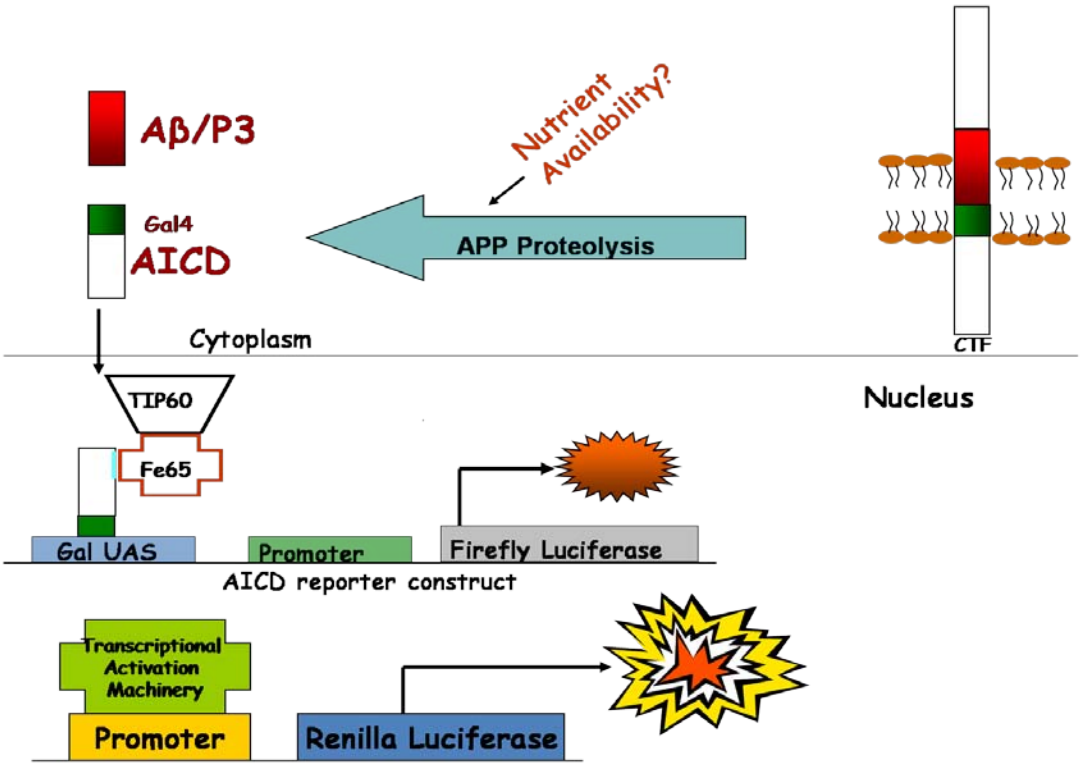
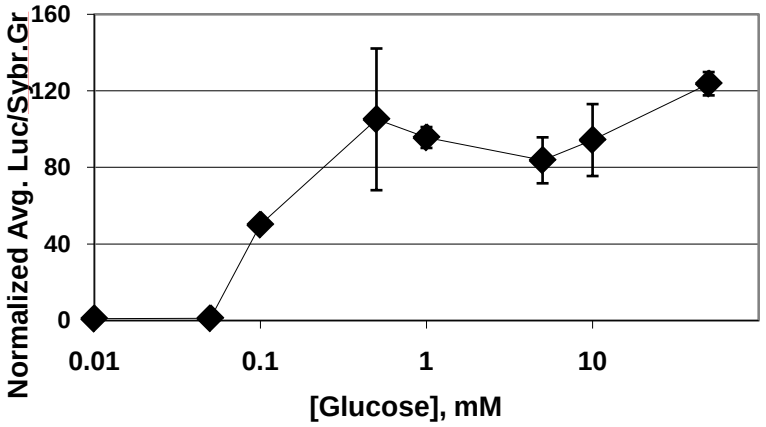
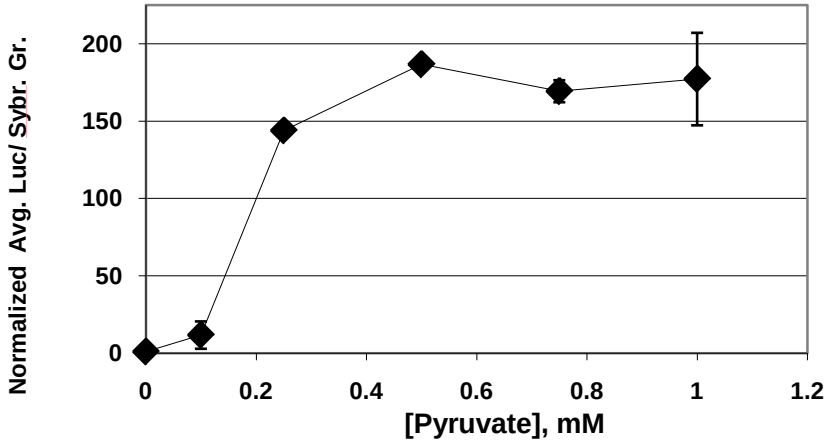


Figure3-2. Glucose and pyruvate modulate AICD levels. SY5Y-APP-Gal4 cells were treated with different concentrations of glucose and Pyruvate dissolved in PBS+0.05% BSA. Lysates were used for luciferase assay. Luciferase CPS were normalized to total protein concentration (μg/μl)

(A) Glucose modulates AICD mediated transactivation



(B) Pyruate modulates AICD mediated transactivation



(C) Glucose and pyruvate specifically decrease AICD mediated transactivation. SY5Y-APP-Gal4 cells were treated with different concentrations of glucose and Pyruvate dissolved in PBS+0.05% BSA. Renilla luciferase plasmid was transiently transfected in cells. Renilla luciferase gene expression is independent of APP processing. Lysates were used for dual-luciferase assay. Firefly-Luciferase CPS were normalized to Renilla luciferase CPS.

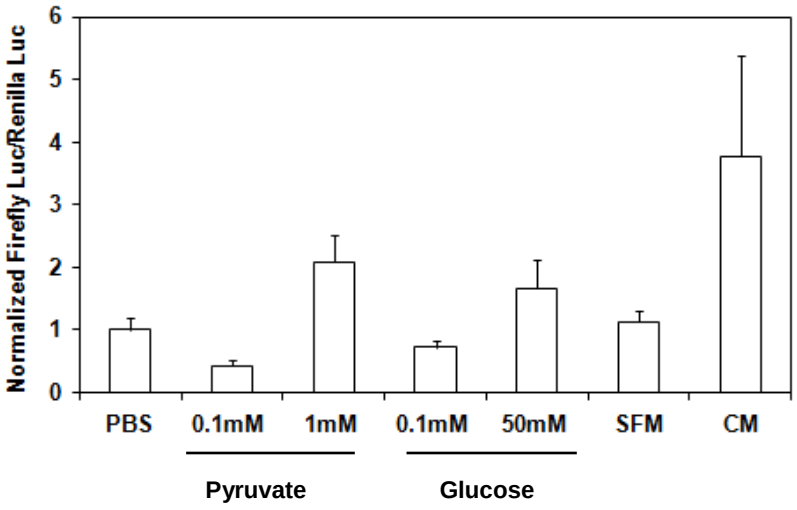
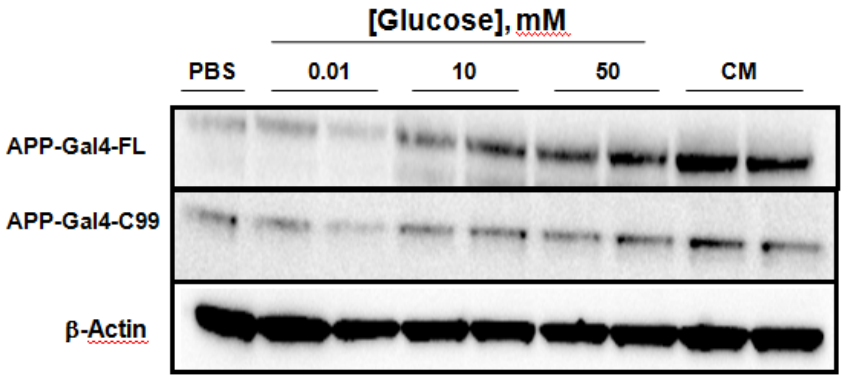
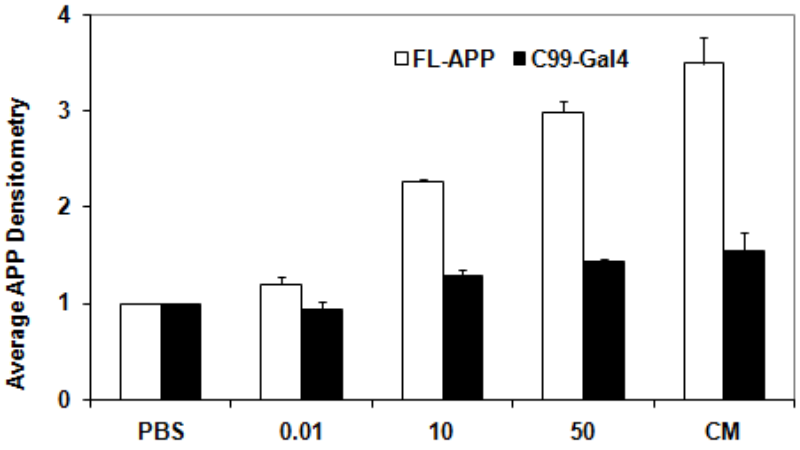


Figure 3-3. Glucose and pyruvate modulate full-length APP levels

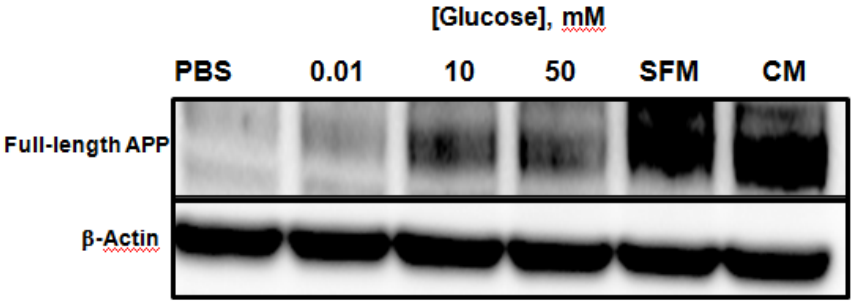
(A) Reduced glucose availability decreases full-length APP levels in SY5Y-APP-Gal4 cells. SY5Y-APP-Gal4 cells were treated with different concentrations of glucose dissolved in PBS+0.5%BSA for 6 hours. Lysates were used for Western Blot. Equal amount of protein (45 μg) was loaded on gel. β-Actin was used as loading control



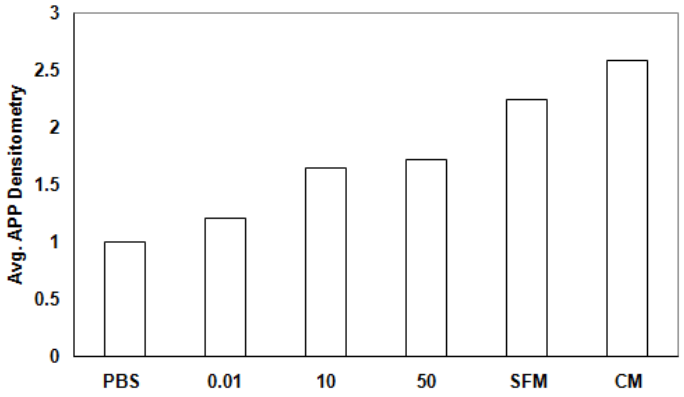
(B) Quantification



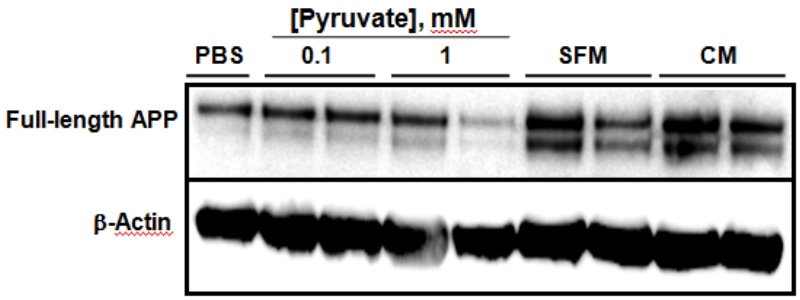
(C) Reduced glucose availability decreases full-length APP levels in Naïve SH-SY5Y cells. . Naïve SH-SY5Y cells were treated with different concentrations of glucose dissolved in PBS+0.5%BSA for 6 hours. Lysates were used for Western Blot. Equal amount of protein (48 mg) was loaded on gel. β-Actin was used as loading control



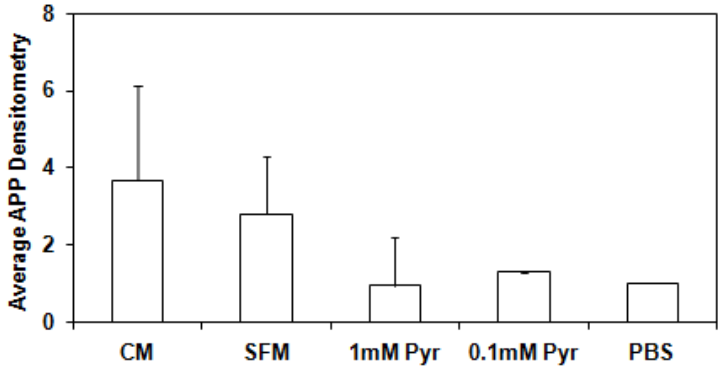
(D) Quantification



(E) Reduced pyruvate availability decreases full-length APP levels in SY5Y-APP-Gal4 cells. SY5Y-APP-Gal4 cells were treated with different concentrations of pyruvatedissolved in PBS+0.5%BSA for 6 hours. Lysates were used for Western Blot. Equal amount of protein (55 μg) was loaded on gel. β-Actin was used as loading control



(F) Quantification



CHAPTER 4

Development and Characterization of a *Drosophila* model of Alzheimer's disease

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Summary

A central event in Alzheimer's disease (AD) is the sequential cleavage of the β-amyloid precursor protein (APP) by β-and γ-secretase enzymes generating the amyloid-beta (Aβ) peptide. Aggregation of Aβ is a major pathological hallmark of AD. It is important to identify regulators of APP processing and Aβ production for developing any therapeutic intervention. Here we have developed and characterized a *Drosophila* model of AD that allows the natural processing of APP by β- and γ-secretase in the central nervous system (CNS) to produce Aβ peptides. We show here that our AD model flies are defective for normal reflexive CNS behavior represented by significantly reduced climbing ability and also display significantly reduced longevity, consistent with the effects of the neurodegeneration we observe in these flies' brains. Further, both of these behavioral defects can be significantly improved by using L-685, 458, consistent with the idea that these phenotypes also require the presence of Aβ peptides and AICD generated through β- and then γ-secretase cleavage of APP. We also show that the Sir2/SirT1 agonist resveratrol, significantly rescued the climbing behavior in our AD flies further confirming that this would be a good model to screen genetic and pharmacologic regulators of APP metabolism.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is considered to be the most common cause of dementia. The pathological features of AD include the presence of senile plaques, neurofibrilary tangles, and massive loss of neurons, primarily in the cerebral cortex and hippocampus. The senile or amyloid plaques are extracellular deposits composed of a small peptide (~4KD) called β-amyloid (Aβ), surrounded by dystrophic neurites, reactive microglia and astrocytes(LaFerla and Oddo 2005). Several lines of evidences lend support to the amyloid hypothesis of AD, according to which Aβ plays the central role in initiating the AD pathogenic cascade(Hardy and Selkoe 2002). Aβ peptides are generated by proteolytic processing from a larger β-amyloid precursor protein (APP) through sequential proteolysis by β- and γ-secretases in amyloidogenic processing pathways(De Strooper and Annaert 2000). In the non-amyloidogenic pathway, APP is cleaved within the Aβ domain by α-secretase, thereby precluding generation of the Aβ peptides(De Strooper and Annaert 2000). APP is cleaved by α-secretase or at a different site by β-secretase, generating the soluble APP ectodomain (sAPPα or sAPPβ) and the two membrane associated C-terminal fragments (CTFα or C83 and CTFβ or C99)(De Strooper and Annaert 2000).These C-terminal fragments then become substrates for the γ-secretase enzyme that cleaves the transmembrane domains, releasing the P3 peptide from the C83 and the Aβ peptide from the C99 along with the APP intracellular domain (AICD) at the cytoplasmic side(De Strooper and Annaert 2000). APP proteolysis is an important step towards development of AD. Therefore, it is important to identify and characterize genes and pharmaceuticals that modulate APP metabolism and Aβ production.

An *in vivo* model is crucial to the study of many disease mechanisms, including AD, as*in vitro*studiesdon't always represent the natural physiology of human cells. As a result, attempts to understand the complex etiology of most diseases have beensignificantly improved through the study of model organisms that serve as surrogates for human patients. The fruit fly, *Drosophila melanogaster*, has been tremendously important and influential in furthering our understanding of the mechanisms of many forms of neurodegenerative diseases, including AD (Fossgreen, Bruckner et al. 1998; Greeve, Kretzschmar et al. 2004; Iijima, Liu et al. 2004; Jeibmann and Paulus 2009; Sarantseva, Timoshenko et al. 2009).

Drosophila model is particularly useful for AD research, as they possess an APP homologue that does not contain the neuropathological Aβ region. As a result, APP expression and processing can be tightly regulated and monitored in a transgenic fly model where human APP is inserted. Additionally, *Drosophila* endogenously produces α-secretase andγ-secretase, but not BACE. As a result, we have employed a transgenic line of flies where we can control the expression of BACE activity. When human APP is expressed in flies, they undergo the same γ-secretase cleavages as in humans (Fossgreen, Bruckner et al. 1998). Since this critical proteolytic cleavage is conserved, and the BACE cleavage can be modulated, *Drosophila* are a strong candidate for the study of AD (Sang and Jackson 2005; Marsh and Thompson 2006).

Many *Drosophila* models of AD utilize transgenic flies that express the toxic form of Aβ, to study its effects on a molecular and behavioral level (Finelli, Kelkar et al. 2004; Iijima, Liu et al. 2004; Crowther, Kinghorn et al. 2005). These models have been useful in highlighting human disease phenotypes, such as amyloid deposits, learning and

memory deficiences, disorientation, and premature death. Though this method of expressing toxic Aβ is useful for modulating the disease phenotype after disease progression has begun, few models have allowed for the natural processing of APP to occur.

Targeted expression of the human genes involved in AD has been used in *Drosophila* models previously, with a focus on both the retina and the nervous system(Greeve, Kretzschmar et al. 2004; Sarantseva, Timoshenko et al. 2009). We report here the characterization of a *Drosophila* model of AD that is both rapid, and sensitive to pharmacological phenotypic modification. Importantly, this model displays very similar pathology to human Alzheimer's patients, including accumulation of Aβ containing plaques in their brains, decreased synaptic brain structures, and memory deficits. However, a significant advantage of our model is that this model develops pathology in roughly 4 weeks, and this pathology can be significantly suppressed by pharmacological intervention.

Results and Discussion

We initially expressed the human forms of the Amyloid Precursor Protein (APP) and β-secretase (BACE) exclusively in the developing fly nervous system using the Gal4/UAS system (Brand and Perrimon 1993). By expressing both APP and BACE together, we are requiring that the natural APP cleavage events mediated by the endogenous α and γ secretases in the fly, and the exogenous human β-secretase provided in our model, must occur in order to generate toxic Aβ42 oligomers normally associated with AD pathology. Further, we hypothesized that this should be sensitive to pharmacological intervetion that affected these enzymes. We utilized the *ELAV-Gal4* reagent to limit the expression of these proteins to the fly nervous system only. We believe that through this specific experimental protocol, we are creating a situation that is more similar to the events that normally lead to the pathology observed in human AD patients.

We initially tested for successful expression of these proteins in adult fly heads through Western Blot analysis (Figure 4-1). To determine that any observed changes in protein expression or migration are due to γ secretase cleavage, we cultured these AD model flies on food containing 100 nM L-685, 458, a strong γ secretase inhibitor. We observe strong expression of APP upon induction with *ELAV-Gal4* in both DMSO and L-685, 458 flies (Figure 4-1A). Further, we observe succesful α-secretase cleavage fragments (C83) in these fly heads (Figure 4-1A, red arrowhead lane 2), a result of the endogenous α-secretase found in flies. In flies cultured in L-685, 458, we observe a build up of the C83 fragment (Figure 4-1A, red arrowhead lane 5), consistent with a blockage of subsequent γ secretase cleavage on the C83 fragment. When we co-express BACE

with APP, we observe a shift in APP processing such that these flies now also produce the C99 fragment (Figure 4-1A, red arrowhead lane 3) indicitive of BACE cleavage of APP. Again, C83 fragments build up in theses flies along withh C99, when cultured in γ secretase inhibitor (Figure 4-1A, bottom arrowhead lane 6). Taken together, these data suggest that we are successfully expressing the human APP and BACE proteins in these fly heads, and that that normal proteolytic cleavage of these proteins succesfully takes place in this model leading to the production of appropriate C-terminal fragments.

As mentioned earlier, Aβ is the major component of the senile plaques found in the brains of AD patients. Therefore it is important to determine if Aβ is generated in our AD model flies through β- and γ-secretase cleavage of APP. We detected $A\beta_{40}$ in APP;BACE flies cultured in DMSO (Figure 4-1B, lane 1). However, the levels of $A\beta_{40}$ were negligible in APP;BACE flies cultured in L-685,458 (Figure 4-1B, lane 2), indicating that γ-secretase activity is inhibited successfully in these flies, as is the production of Aβ. There was an 80% decrease in $A\beta_{40}$ levels in the L-685,458 treated flies, compared to the DMSO treated flies (Figure 4-1C). C99 is the substate for γ-secretase cleavage in the amyloidogenic pathway of APP processing. An inhibition of γ-secretase activity should lead to a build up of C99, along with a decrease in Aβ levels. We next quantified the levels of C99 in these APP;BACE fly heads and observed that there was a 30% increase of C99 in the flies culutured in L-685,458 (Figure 4-1B, lane 2; Figure 4-1C) compared to those cultured in DMSO (Figure 4-1B, lane 1; Figure 4-1C). Taken together, these results suggest that we have sucessfully recapitulated the amyloidogenic pathway of APP processing in the APP; BACE flies.

We expressed APP and BACE continuously during development to determine if this genotype could produce viable flies with prominent phenotypes that were consistent with nervous system degeneration. Upon eclosion, we initially observed two distinct morphological abnormalities in these flies: crumpled wings (Figure 4-2B), and the presence of melanotic masses on both the abdomen and proboscis of the fly (arrows in Figure 4-2B). Abnormal wing development was previously observed in flies expressing human APP in fly wings (Fossgreen, Bruckner et al. 1998), though melanotic tissue mass has not been previously described, and is due to localized buildup of pigment in the presence of tissue damage and/or necrotic tissue in the area. These phenotypes are observed in flies expressing human APP alone, but are greatly enhanced by flies that express both the human APP and BACE proteins (Figure 4-2C), consistent with the idea that the phenotypes are dependent upon the expression of BACE, and may be due to increased accumulation of Aβ42 oligomers. Both of these phenotypes are strongly suppressed when these flies are cultured on L-685, 458 (Figure 4-2C), strongly suggesting that proper γ secretase activity is also required for these phenotypes.

We next analyzed adult brains from our model flies. Gross anatomical comparison between wild type brains (Figure 4-3A) and brains from flies expressing the APP and BACE proteins (Figure 4-3B) showed severe degeneration in a number of brain structures, including the mushroom body, the antennal lobes, and the optic lobes. To determine the effects on synaptic structures of neurons involved in learning and memory in the fly, we analyzed the soma, dendrites, and axons of the Kenyon cell neurons (Heisenberg, Borst et al. 1985). We co-expressed a membrane tagged form of GFP (CD8-GFP) in our AD fly background in order to visualize whole brain anatomy fluorescently.

We found a significant reduction in the size of the Calyx (the dendritic field) and the mushroom body lobes (the axonal bundles) in flies that express both APP and BACE as compared to control flies, or flies that express APP alone (Figures 4-3C, D). Though there is no significant difference in the size of the Kenyon Cell neuronal clusters, there is a trend that shows that these structures are also reduced compared to both control flies and flies that express APP alone (Figure 4-3D). Because these fly brains were dissected at day 6 after eclosion, it may be that we are observing those structures most sensitive to initial degeneration (dendrites and axons), and that we would observe a signficant difference in Kenyon cell neuronal cluster size if we were to analyze these flies at a later age. When flies expressiong both APP and BACE are cultured on media containing L-685, 458, we observe a significant increase in the size of synaptic structures measured compared to control flies cultured on DMSO (Figure 4-3E). There is an increase in the overall neuronal population of Kenyon cells (as measured by the increase in surface area of Kenyon cell soma) as well as a significant increase in the Kenyon cell dendritic fields of these flies (Figure 4-3E). Again, though there is no significant increase in axonal fields between AD flies cultured on DMSO compared to L-685, 458, there is a strong trend towards a larger axonal field. Taken together, these data suggest that those structures involved in synaptic function (dendrites and axons) in Kenyon neurons are smaller in overall size in flies expressing APP and BACE compared to controls. Further, this reduction in size is dependant upon both the expression of human BACE, and the proper function of the γ secretase complex, consistent with the idea that these phenotypes require the presence of Aβ peptides.

In order to validate that the reduction in dendritic and axonal structures observed in our AD model correlates with the presence of amyloid plaques, we stained these brains with Thioflavin S as has been previously described by Iijima et al (Iijima, Liu et al. 2004). We detected a significant number of Thioflavin S positive puncta in brains that express APP and BACE compared to controls (Figure 4-4A compared to 4-4B). We further observed that there was a decrease in the number of Thioflavin S positive puncta in the brains of flies expressing APP and BACE when cultured on L-685,458 media as compared to flies expressing APP and BACE when cultured on DMSO (Figure 4-4C compared to 4-4D). Taken together, these data indicate that reduction in the size of the neuroanatomical structures measured in fly brains correlates well with the presence of amyloid.

To further confirm the presence of amyloid plaques in the brains of the AD model flies, we stained the brains of flies expressing APP and BACE with antibodies (6E10) that specifically recognize amyloid-beta, as previously described by Chaing et al (Chiang, Iijima et al. 2009). We observed a significantly larger number of 6E10 positive plaques in the brains of flies cultured on DMSO (Figures 4-4E, 4-4G) as compared to flies cultured on L-685, 458 (Figures 4-4F, 4-4G). These results further confirm the presence of amyloid-beta positive plaques in the brains of our AD model flies, and show that these plaques are sensitive to pharmacological inhibition of γ secretase.

As an initial test of central nervous system function in our model, we utilized a simple, yet powerful behavioral assay, the climbing assay (Le Bourg and Lints 1992). This assay has been previously used to successfully test for nervous system function in fly models of multiple diseases, including Alzheimer's Disease (Iijima, Liu et al. 2004).

Briefly, flies display a negative geotaxis response when given a mechanical stimulus. When tapped to the bottom of a vial, flies normally can orient themselves rapidly, and will begin climbing to the top of the vial. By assaying the fly's ability to perform this response, we are able to compare broad nervous system function of reflex behavior within flies of different genotypes. When cultured on normal food, flies that express both APP and BACE show a significant decrease in their climbing ability compared to bothcontrol flies, and flies that express APP alone (Figure 4-5A). This behavior also decreases with age, but is apparent within the first 10 days of the assay (Figure 4-5A). Further, on normal food, the longevity of flies that express APP alone, and APP with BACE is significantly decreased compared to control flies (Figure 5B). When cultured on food that contains L-685, 458, the decrease in both climbing behavior and longevity is significantly rescued and is comparable to control flies (Figures 5C, D). Taken together, these data suggest that our AD model flies are defective for normal reflexive CNS behavior and also display significantly reduced longevity, consistent with the effects of the neurodegeneration we observe in these flies' brains. Further, both of these phenotypes can be significantly improved by using L-685, 458, consistent with the idea that these phenotypes also require the presence of Aβ42 oligomers.

One of the earliest symptoms of Alzheimer's disease is memory loss. To test for deficits in learning and memory in our AD model, we performed the conditioned courtship suppression assay (Siegel and Hall 1979). This assay is an associative conditioning procedure that is ethologically based and capable of measuring both learning and memory in individual flies(Broughton, Tully et al. 2003). Briefly, courting behavior by males in *Drosophila* follows a linear, stereotyped, and well documented set

of behaviors, and this behavior is modified by previous sexual experience (Siegel and Hall 1979; Hall 1994). Courtship conditioning is a form of associative learning in *Drosophila*, where male courtship behavior is modified by exposure to a previously mated female that is unreceptive to courting (Siegel and Hall 1979; Siwicki, Riccio et al. 2005).Thus, after 1 hour of courting a mated female, males suppress their courtship behavior even towards subsequent receptive virgin females for 1-3 hours by 40% or more(Siegel and Hall 1979; Joiner Ml and Griffith 1997; Kane, Robichon et al. 1997; Kamyshev, Iliadi et al. 1999).

To determine effects on learning in AD flies,male flies were placed in a courtship chamber with a previously mated (unreceptive) wild type female for 60 minutes. The amount of time the male spent performing courtship behavior was assessed during the first 10 minutes of this training and compared to the last 10 minutes of the training period. Wild type control flies showed a significant drop in courtship behavior in the last 10 minutes of training as compared to the first 10 minutes of training (Figure 4-6A), indicative of an appropriate learning response. Flies that express APP and BACE also showed an appropriate learning response regardless of whether these flies were cultured on DMSO or L-685, 458 (Figure 4-6A). Importantly, this indicates that our AD model flies are able to successfully perceive and interpret the sensory stimuli in this assay normally, and that they are able to alter their behavior appropriately (learn) in response to training. There have been five phases of memory defined in *Drosophila*, immediate recall (0-2 minutes post-training), short term memory (out to 1 hour post-training), medium term memory (out to six hours), anesthesia-resistant memory (out to two days), and long term memory (out to 9 days) (Greenspan 1995; McBride, Choi et al. 2005). We

assayed our model flies for immediate recall memory by transferring trained male flies to clean mating chambers with a receptive virgin female within 2 minutes of training. We then assayed their courtship behavior. Trained wild type males showed a clear decrease in courtship activity as compared to parallel sham trained flies (Figure 4-6B), indicating a change in behavior consistent with normal immediate recall memory of training. However, AD flies cultured on DMSO showed no significant decrease in courtship behavior within 2 minutes of prior training compared to sham trained AD flies cultured on this media (Figure 4-6B), indicating that though these flies are capable of learning, they are deficient in their immediate recall memory of this learning. However, culturing AD flies on L-685, 458, showed a clear decrease in courtship activity as compared to parallel sham trained AD flies cultured on this media (Figure 4-6B), indicating that the drug L-685, 458 can suppress the immediate recall memory defect normally associated with AD flies cultured on DMSO. This is interesting to note, particularly as culturing flies on L-685, 458 does not fully rescue the decreased Kenyon neuron morphology in our AD flies.

Alzheimer's disease is a disease of aging. Numerous studies in model organisms have shown that Sir2/SirT1the NAD^+-dependent deacetylase, is a critical regulator of the aging process (Anderson, Bitterman et al. 2003; Howitz, Bitterman et al. 2003; Cohen, Miller et al. 2004; Rogina and Helfand 2004). Both genetic and pharmacologic induction of SirT1 has also shown to be beneficial in many *in vivo* and *in vitro* models of AD. The SirT1 agonist resveratol has been shown to lower the levels of secreted and intracellular Aβ peptides produced from different cell lines (Marambaud, Zhao et al. 2005). Based on these studies, we wanted to determine if our model was sensitive to test the efficacy of

the Sir2/SirT1 agonist resveratrol using the climbing behavior assay in our AD model flies. We found that flies cultured on media containing 100 nM resveratrol significantly rescued the climbing behavior in our AD flies (Figure 4-7), further confirming that this model was sensitive to pharmacological intervention beyond L-685, 458.

To summarize, our results show that we have successfully created an AD model in *Drosophila* that is both rapid, and sensitive to pharmacological intervention. Our results specifically show that these AD model flies can recapitulate amyloidogenic proteolytic processing of APP by β- and γ-secretase respectively, leading to the production of Aβ. We have shown that presence of Aβ in the central nervous system of these flies can recapitulate some of the pathological, neuroanatomical and behavioral changes seen in AD patients. We have further shown that some of these changes can be rescued by the γ-secretase inhibitor L-685,458 and the Sir2/SirT1 agonist resveratrol. We suggest that this model will serve as a powerful tool for future screening of genetic and pharmacologic modulators of APP proteolysis and Aβ production.

Materials and Methods

***Western Blot Analysis*:** For Western blot analysis**,** 5-10 fly heads were collected from respective genotype and lysed in RIPA Buffer containing different protease inhibitors [Antipain(100μM), Aprotinin (2 μg/ml), Benzamide (15 μg/ml), Chymostatin (100 μM), Leupeptin (100 μM), Pepstatin A (1μM), PMSF (1μM), Sodium Metabisulfite(0.1 nM). 1 μl of lysates were used for protein assay with the help of BCA Protein Assay Kit (Pierce, Inc.). The BCA protein utilizes a standard curve generated by RIPA standards (different dilutions of BSA protein in RIPA buffer). According to the protein concentrations, samples for Western Blot were prepared using the 4x Nupage LDS sample buffer (Invitrogen, Inc.) containing 0.2% BME (β-Merkaptoethanol, Sigma Aldrich). Equal amounts of protein were loaded on to each well of NuPAGE 4-12% Bis Tris Gel. From the gel the proteins were transferred on to 0.25 μ PVDF (Immobilon FL) membrane (Millipore) using a semi-dry transfer apparatus. Blots were probed with different antibodies and the target protein densitometry was normalized to b-actin densitometry using Odyssey Infrared Imaging system (LI-COR Biosciences).

Immunohistochemistry and Antibodies: APP C-terminal antibody (A8717; Sigma Aldrich, Inc), monoclonal anti β-Actin (A5441, Sigma Aldrich, Inc), APP 6E10 (ab10146, Abcam), goat anti-Rabbit IR-Dye800 CW (926-3211) and/or Goat anti-Mouse IR Dye 680 (926-3200) were used as secondary antibodies.

Adult and larval brains were dissected, fixed and prepared essentially as described(Tio and Moses 1997). Adult and larval brains were dissected directly in fix. Brains were mounted in vectashield (Vector Labs, H-1000). All fluorescent imaging was done using an Olympus FluoView FV1000 laser scanning confocal microscope.

Secondary antibodies for immunohistochemisrty used were goat anti-mouse TRITC (# 115-116-072, 1:150), goat anti-rabbit TRITC (# 111-116-144, 1:250), goat anti-rabbit Cy5 (#111-176-144, 1:1000), goat anti-mouse Cy5 (# 115-176-072, 1:500). All secondary antibodies were from Jackson ImmunoResearch.

Thioflavin S staining was performed as described (Iijima, Chiang et al. 2008).

Pharmacologic reagents used: Resveratrol (Sigma Aldrich) was used for some experiments. Resveratrol was dissolved in DMSO. 100 nM resveratrol was used for preparing food vials for AD model flies. γ-secretase transition state inhibitor, L-685,458, was purchased from Sigma Aldrich. 100 nM L-685,458 was used for preparing food vials for AD model flies.

Drosophila Stocks and Genetics: All crosses were carried out at 25°C. Normal food consisted ofa standard cornmeal, yeast, molasses recipe as follows: 120g cornmeal (LabScientific FLY-8009-10), 48g yeast (LabScientific 8030-5), 9g agar, 120ml molasses (LabScientific FLY-8008-4), 24ml Tegosept (10% w/v methyl p-hydroxybenzoate in 95% ethanol), and 9.5 ml Propionic Acid) with 840 ml of water. Drug food was prepared adding drug to 17 ml of water and mixing thoroughly. Cornmeal, yeast, agar, molasses, tegosept, and propionic acid were then added to a final volume of 30 ml, and food was prepared as normal. Flies were cultured on drug food for their entire lifespan from embryogenesis to death.

The Gal4/UAS system was used for the overexpression of UAS transgenes in *Drosophila* as described (Brand and Perrimon 1993). BL# refers to Bloomington Stock Center stock number. Stocks used are described: *UAS:APP; UAS:BACE* (Greeve, Kretzschmar et al. 2004), *P{GawB}elav*^{C155}(*ELAV-Gal4*, BL#458), P{GawB}elav^{C155},

P{UAS-mCD8::GFP.L}LL4, P{hsFLP}1, w^{-}(BL#5146), w^{1118}(BL#3605). Wild type flies used were Canton S.

Behavioral testing and training: For climbing assays, a modified version of Le Bourg and Lints was used (Le Bourg and Lints 1992).Flies were collected between 0-8 hours after eclosion and assayed every two days. Groups of 10 or fewer flies were transferred to a clean, empty vial and given 18 seconds to climb 5 cm. The number of flies that successfully reach the 5 cm line are recorded.

For courtship behavioral training, virgin male flies of the appropriate genotype were collected between 0 and 6 hours after eclosion and transferred to individual food vials (with or without drug as appropriate). All flies were maintained at 25°C in a 12:12 light:dark cycle at 60% humidity. All behavioral tests were performed in a separate room maintained at 25°C and 60% humidity and illuminated under a constant 130 V white light Kodak Adjustable Safelight Lamp mounted above the courtship chambers. All behavior was digitally recorded using a Sony DCR-SR47 Handycam with Carl Zeiss optics. Subsequent digital video analysis of time spent performing courtship behavior was quantified using iMovies software (Apple). The total time that a male performed courtship activity was measured and scored. The Courtship Index (CI) was calculated as the total time observed performing courting behavior divided by the total time assayed, as described (Siegel and Hall 1979).

Virgin female wild type (Canton S) flies were collected and kept in normal food vials in groups of 10. Male flies were aged for 3 days before behavioral training and testing. All tests were performed during the relative light phase. Mated Cantons S females used for training were 5 days old, and observed to have mated with a Canton S

male the evening prior to training. Virgin female Canton S targets used were 4 days old. Male flies were assigned to random groups the day of training, and assays were set up and scored blind. Male flies were transferred without anesthesia to one half of a partitioned mating chambers from Aktogen (http://www.aktogen.com) that contained a previously mated Canton S female in the other partitioned half. Males were allowed to acclimate for 1 minute, then the partition between the male and female was removed. Male flies were then trained for 60 minutes. After 60 minutes, male flies were transferred within 2 minutes without anesthesia to one half of a partitioned mating chamber that contained a virgin Canton S female in the other partitioned half. The partition was removed and the flies were recorded for 10 minutes.

Figure legends

Figure 4-1. (A) Western Blot Analysis detecting full-length APP and C-terminal fragments of APP in AD model flies exposed to DMSO (vehicle control) and L-685,458. Control flies (Elav out-crossed to w-) and AD model flies were collected from respective crosses on DMSO (vehicle control) and L-685,458. These flies were maintained in appropriate drug food for 6 days. At 6 days age, 6-7 fly heads were collected from respective vials. After protein assay, equal amounts of protein (~ 45 μg) were loaded on the gels. Fly β-Actin was used as the loading control. (B) Western Blot analysis for detecting $A\beta_{40}$ and APP-CTFβ (C99) in AD model flies expressing both APP and BACE exposed to DMSO (vehicle control) and L-685,458. AD model flies expressing both AP and BACE were collected from respective crosses on DMSO (vehicle control) and L-685,458. These flies were maintained in appropriate drug food for 6 days. At 6 days age, 6-7 fly heads were collected from respective vials. After protein assay, equal amounts of protein (~ 45 μg) were loaded on the gels. Fly β-Actin was used as the loading control. (C) Quantification of panel B blot shows an80% decrease in $A\beta_{40}$ levels and a 35% increase in C99 levelsin APP and BACE expressing fly heads exposed to L-685,458 compared to those exposed to DMSO.

Figure 4-2. External morphology of AD model flies (A) Control fly (Elav out-crossed to w-) shows normal external phenotype and (B) AD model fly expressing both APP and BACE shows necrosis, marked by the presence of melanotic masses on both the abdomen and proboscis, and crumpled wings. (C) Quantification of the occurrence of necrosis and crumpled wings in AD model flies shows that the g-secretase inhibitor L-685,458

decreases the occurrence of necrosis and crumpled wings in AD model flies. Error bars represent standard deviation and p-value was <0.05.

Figure 4-3. Gross anatomical comparison of AD model fly brains(A) wild type brains and (B) brains from flies expressing the APP and BACE proteins were dissected out at 6 days age. (C) Atrophy of Kenyon cells (soma), Calyxes (dendrites) and Lobes (axons) in AD model fliesexpressing both APP and BACE (third panel) compared to WT (first panel) and APP expressing (second panel) fly brains. (D) Areas of Kenyon cells, Calyxes and Lobes were measured as indicated in (C) and presented as averages± standard deviation (n = 3 hemispheres). Asterisks indicate significant differences from control (ELAV) and Elav;APP ($p<0.05$, Student's t-test). (E) Areas of Kenyon cells, Calyxes and Lobes of AD model flies expressing both APP and BACE (Elav; APP; BACE) raised on vehicle control (DMSO) and L-685,458were measured as indicated in (C) and presented as averages±Standard deviation (n = 3 hemispheres).

Figure 4-4. Immunostaining of AD model fly brains at Day 6 age(A,B,C.D) Thioflavin S (TS) staining of brains of 6 days old flies. No signal was detected in the control (A). (B) Arrowheads and arrows indicate TS-positive deposits in Elav; APP; BACE fly brains. (C) TS positive deposits abundant in Elav; APP; BACE fly brains (at day 6) raised in DMSO. (D) TS positive deposits less abundant in Elav; APP; BACE fly brains (at day 6) raised in L-685,458. (E-F) 6E10 staining of brains of 6 days old Elav; APP; BACE flies (E) 6E10 positive plaques abundant in Elav; APP; BACE fly brains (at day 6) raised in DMSO. (F) 6E10 positive plaques much less abundant in Elav; APP; BACE fly brains (at day 6) raised in L-685,458. (G) Numbers of 6E10 positive plaques were presented as

averages±SD (n = 4 hemispheres). Asterisks indicate significant differences DMSO ($p<0.05$, Student's t-test).

Figure 4-5. Climbing ability and Longevity of AD model flies (A) Climbing ability of AD model flies raised in normal food. Climbing assay was performed on AD model flies every other day after eclosing till 30 days age. Bars represent the percentage of flies that climbed to the top of the vial at 18 seconds after knocking flies to the bottom are shown (average± Standard deviation (n = 50-100 for each genotype)). Elav; APP; BACE flies showed a decrease ($p<0.05$) in climbing ability (B) Longevity of AD model flies raised in normal food. The percentage of flies surviving was plotted against the age in days. Elav; APP; BACE flies showed a strong decrease ($p<0.05$) in longevity. (C) Climbing ability of Elav;APP;BACE flies raised in DMSO (vehicle control) and L-685,458. Climbing assay was performed on these flies every other day after eclosing till 30 days age. Bars represent the percentage of flies that climbed to the top of the vial at 18 seconds after knocking flies to the bottom are shown (average ± Standard deviation (n = 50-100 for each drug type)). Elav; APP; BACE flies raised in L-685,458showed anincrease ($p<0.05$) in climbing ability compared to the flies raised in DMSO (D) Longevity of AD model flies raised in normal food. The percentage of flies surviving was plotted against the age in days. Elav; APP; BACE flies showed raised in L-685,458showed anincrease ($p<0.05$)in longevity.

Figure 4-6. Learning and memory behavior of AD model flies (A) Courtship learning assessment on AD model flies.The amount of time the male spent performing courtship behavior was assessed during the first 10 minutes of this training and compared to the last 10 minutes of the training period. Wild type control flies, Elav;APP;BACE flies raised in

DMSO and Elav;APP;BACE flies showed a significant drop in courtship behavior in the last 10 minutes of training as compared to the first 10 minutes of training. (B) Immediate recall memory assessment in AD model flies. Elav; APP; BACE flies raised in DMSO showed decrease in immediate recall memory compared to Elav; APP; BACE flies raised in L-685,458 or control flies.

Figure 4-7. Climbing ability of Elav;APP;BACE flies raised in DMSO (vehicle control) and resveratrol. Climbing assay was performed on these flies every other day after eclosing till 30 days age. Bars represent the percentage of flies that climbed to the top of the vial at 18 seconds after knocking flies to the bottom are shown (average ± Standard deviation ($n = 50\text{-}100$ for each drug type)). Elav; APP; BACE flies raised in resveratrol showed anincrease ($p<0.05$) in climbing ability compared to the flies raised in DMSO

Figures

Figure 4-1A. Western Blot Analysis detecting full-length APP and C-terminal fragments of APP in AD model flies exposed to DMSO (vehicle control) and L-685,458.

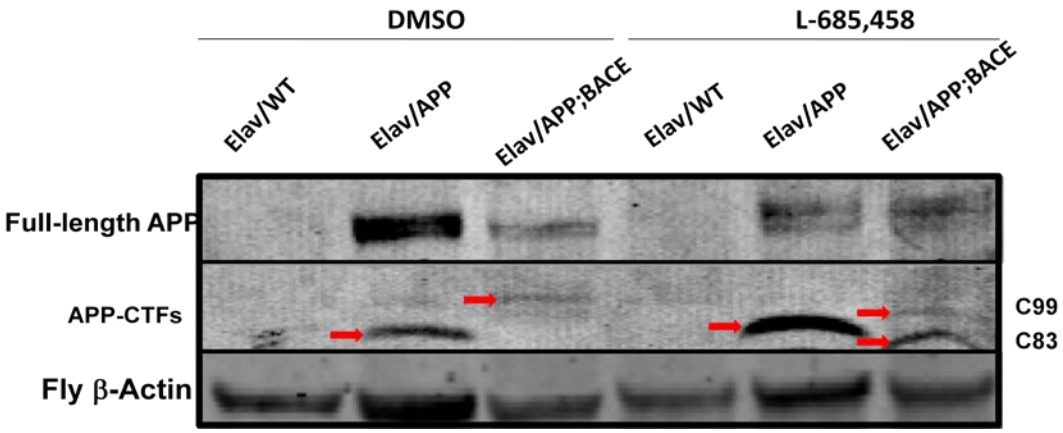


Figure 4-1 B. Western Blot analysis for detecting $A\beta_{40}$ and APP-CTFβ (C99) in AD model flies expressing both APP and BACE exposed to DMSO (vehicle control) and L-685,458.

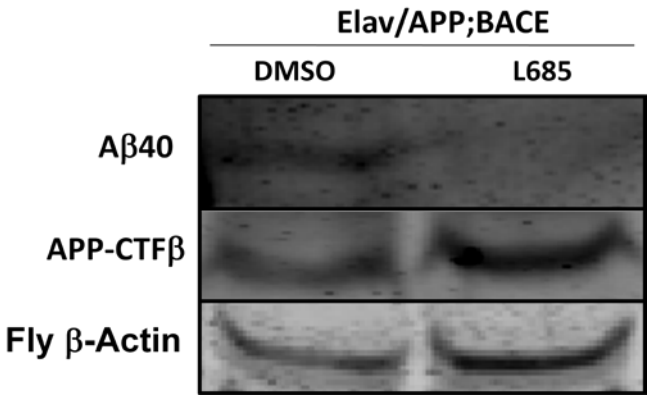


Figure 4-1 C. Quantification of $A\beta_{40}$ and C99 levels in Elav/APP;BACE flies exposed to vehicle control (DMSO) and L-685,458.

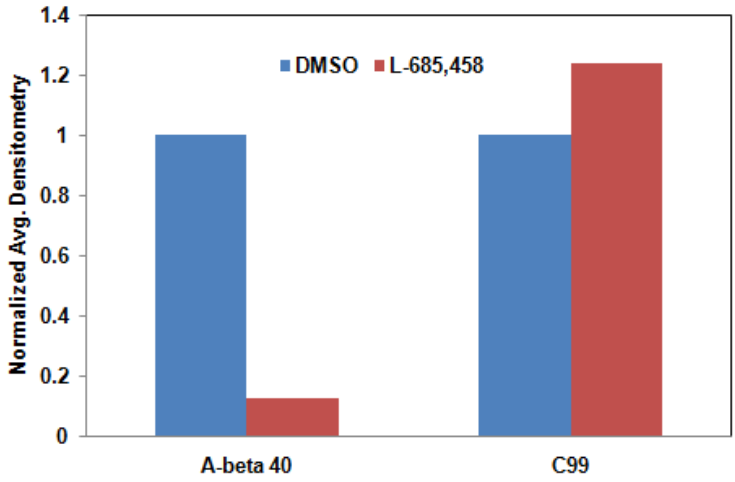
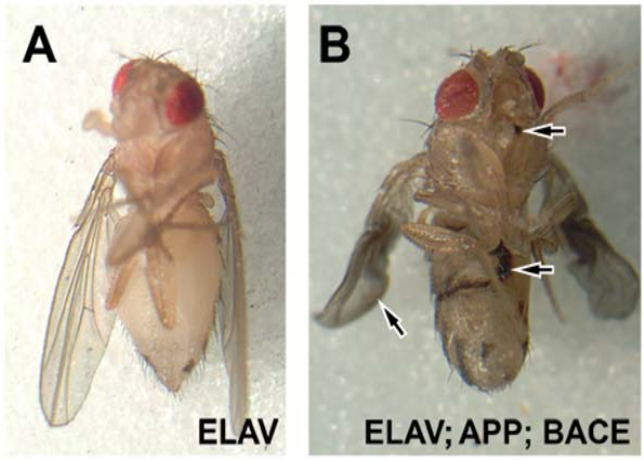


Figure 4-2. External morphology of AD model flies.



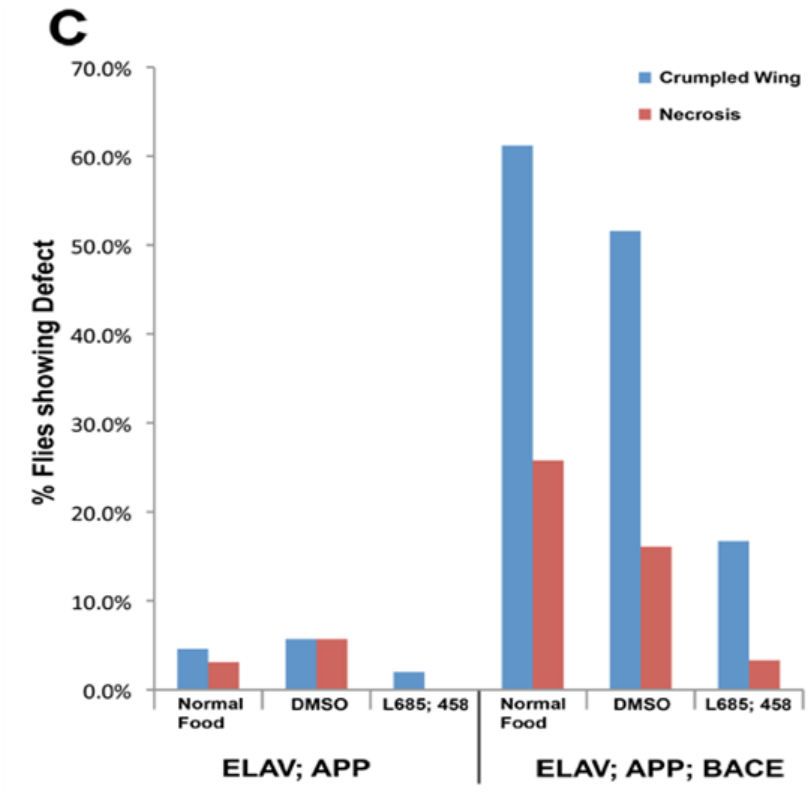
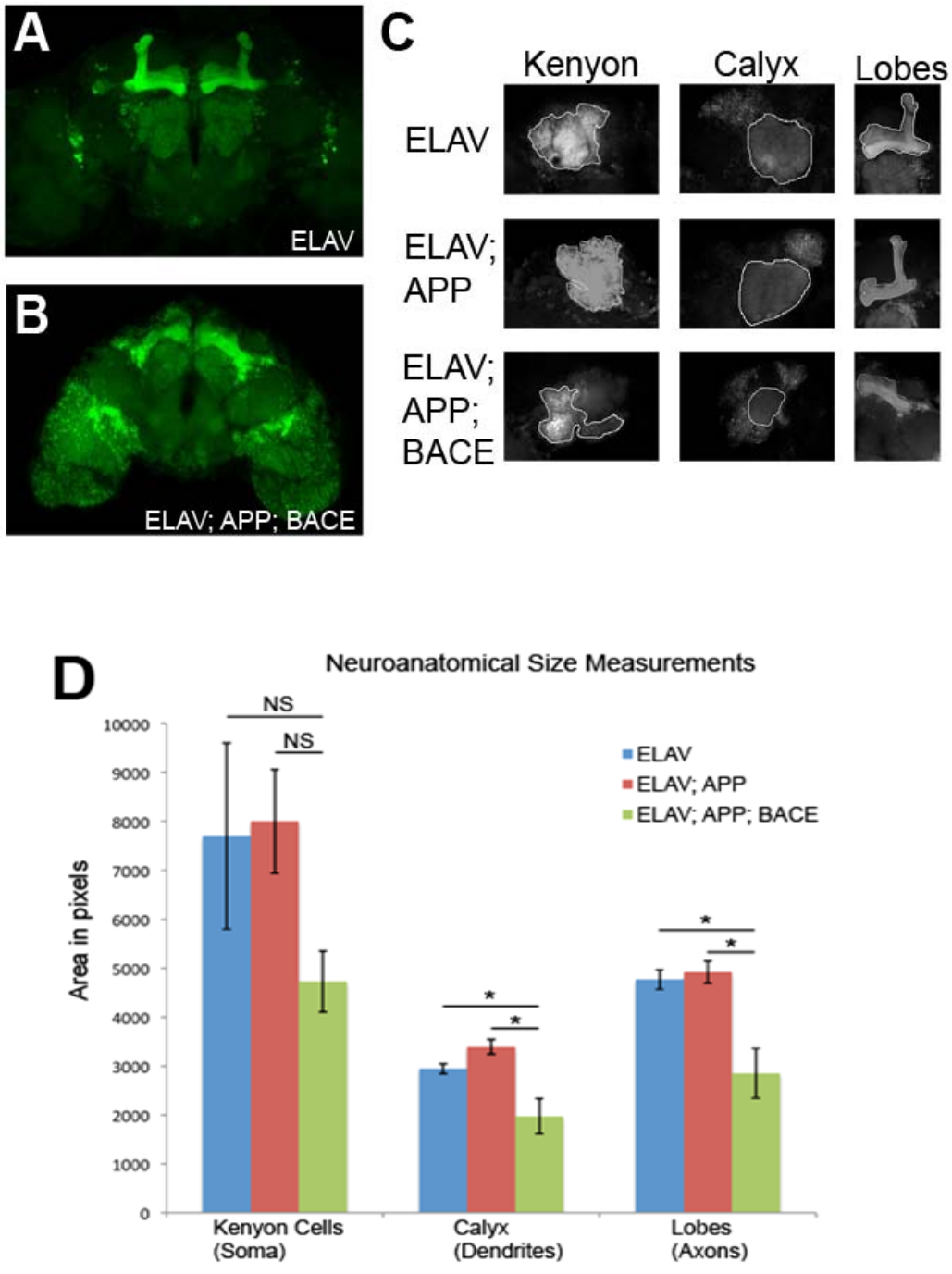


Figure 4-3. Expression of APP and BACE in the fly brains affect the neuroanatomical structures



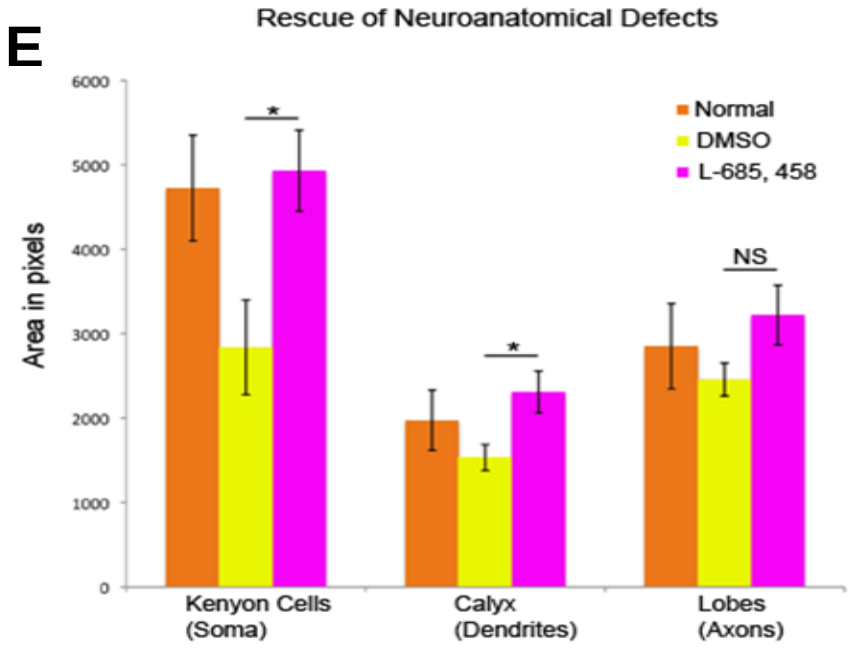

E
Rescue of Neuroanatomical Defects
Area in pixels
6000
5000
4000
3000
2000
1000
0
Normal
DMSO
L-685, 458
*
*
NS
Kenyon Cells
(Soma)
Calyx
(Dendrites)
Lobes
(Axons)

Figure 4-4. Thioflavin S and 6E10 staining of AD model fly brains expressing both APP and BACE

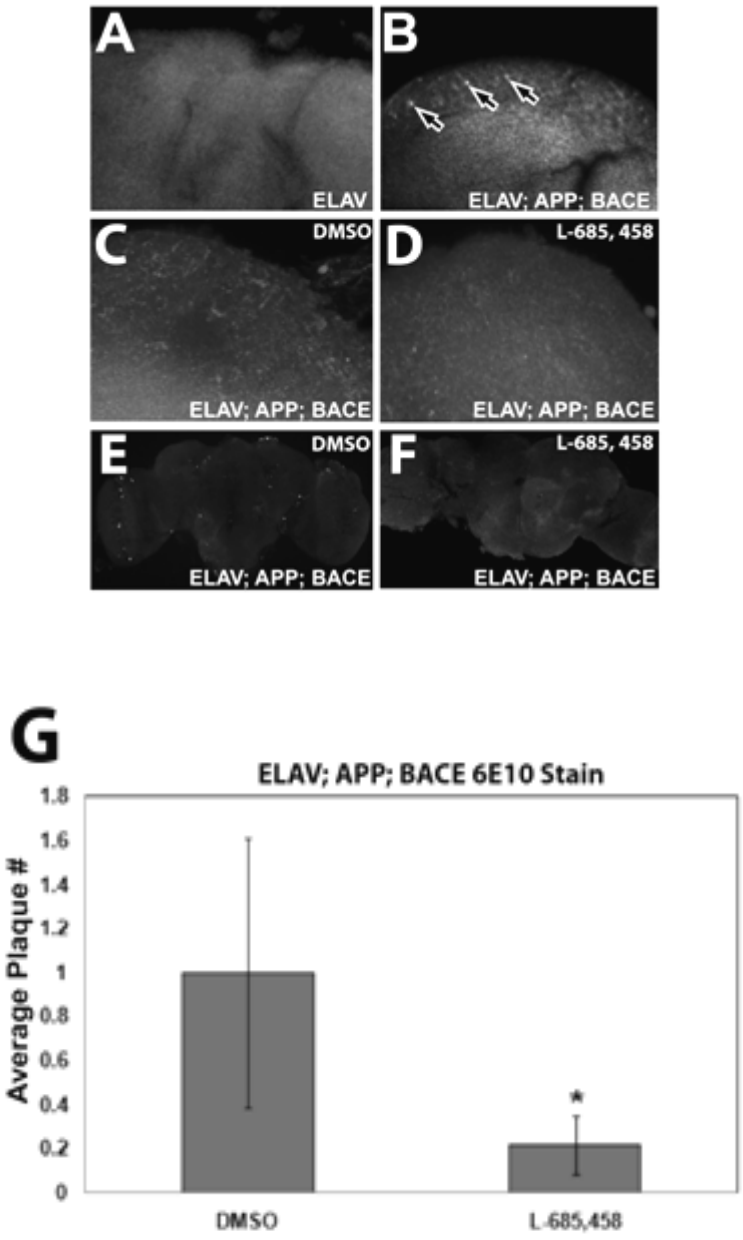
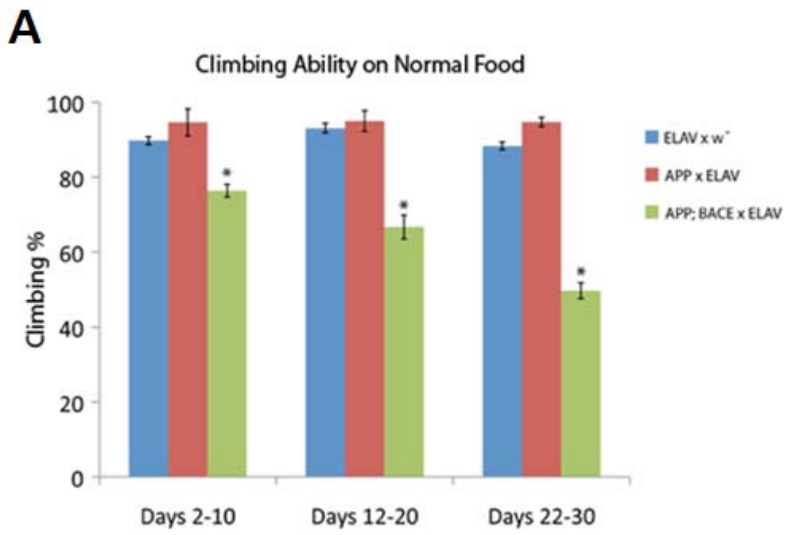


Figure 4-5A and 5B. Climbing ability and Longevity of AD model flies



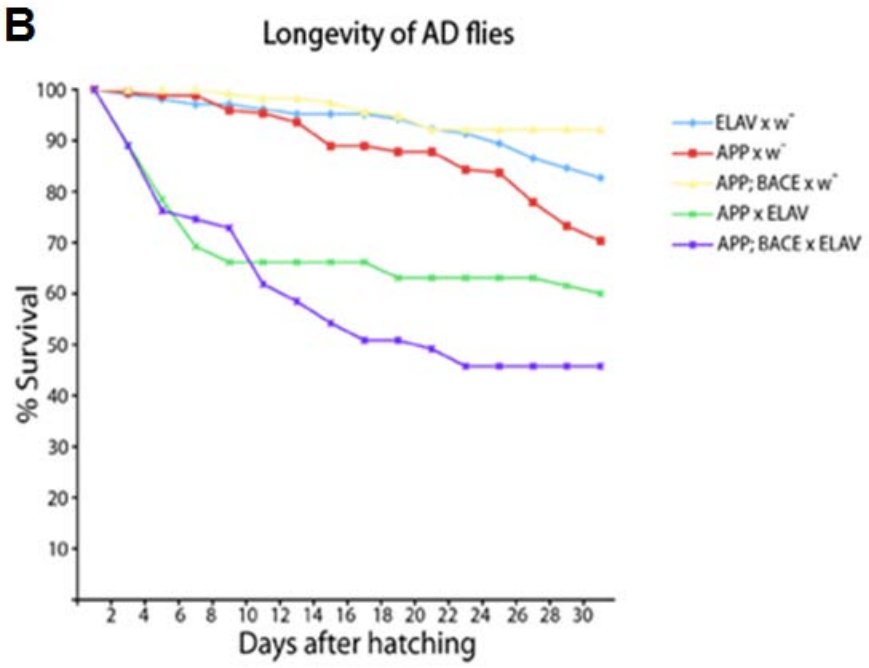
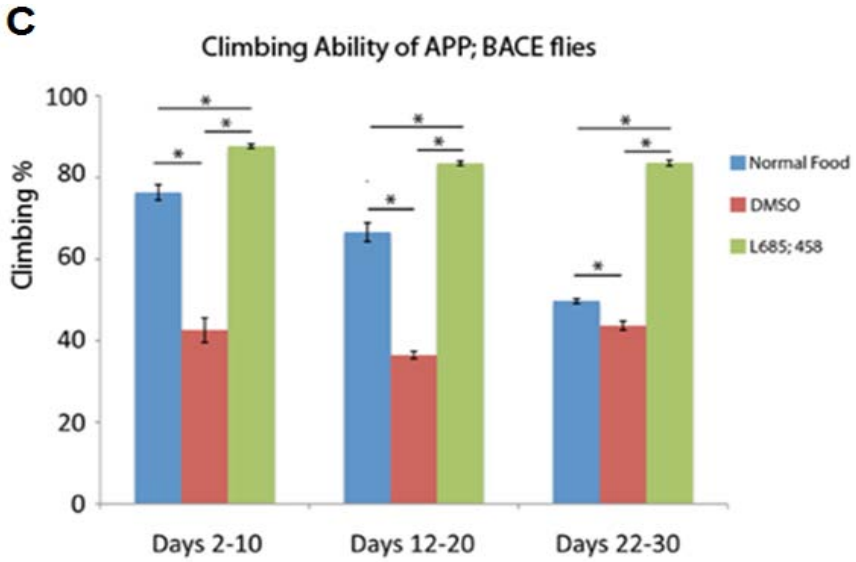


Figure 4-5C and 5D. Climbing ability and longevity of AD model flies expressing APP and BACE exposed to drug food.



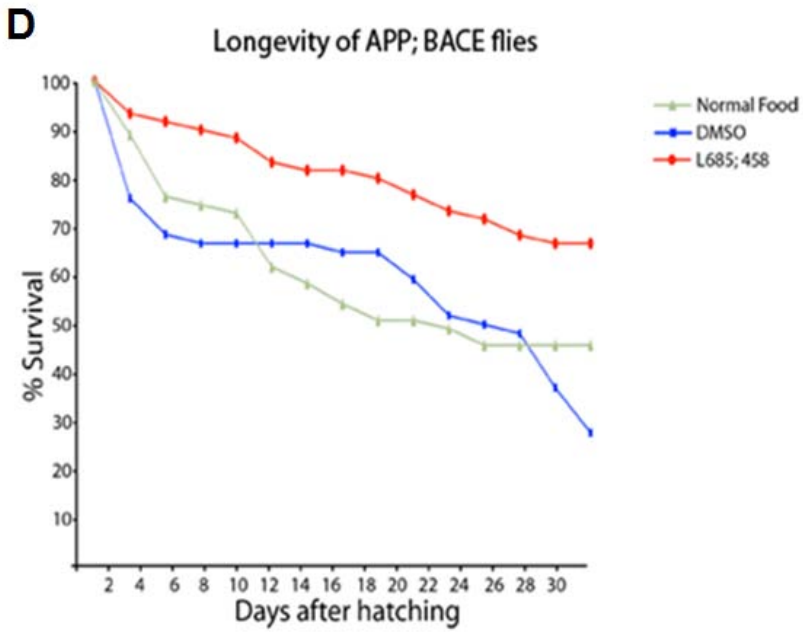


Figure 4-6. Learning and memory test on AD model flies

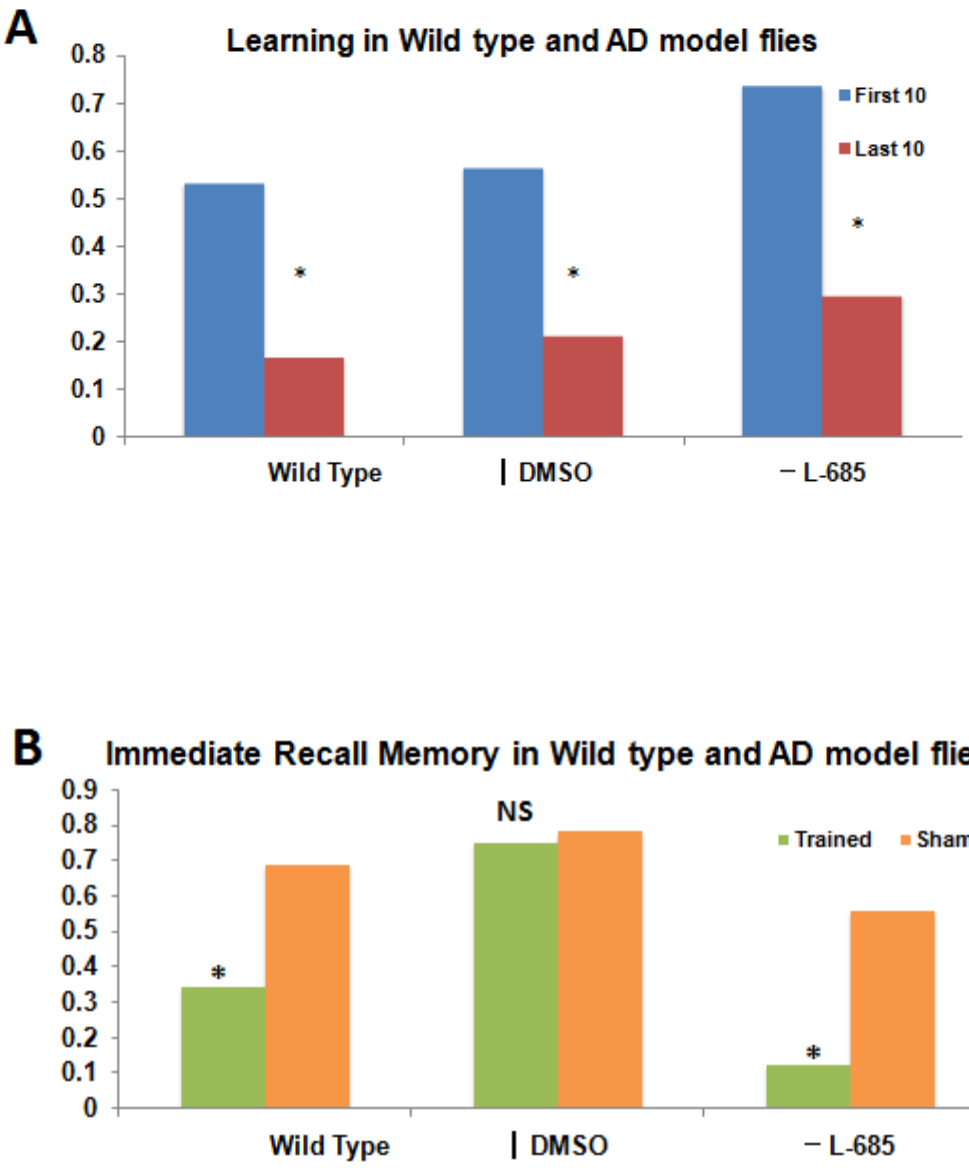
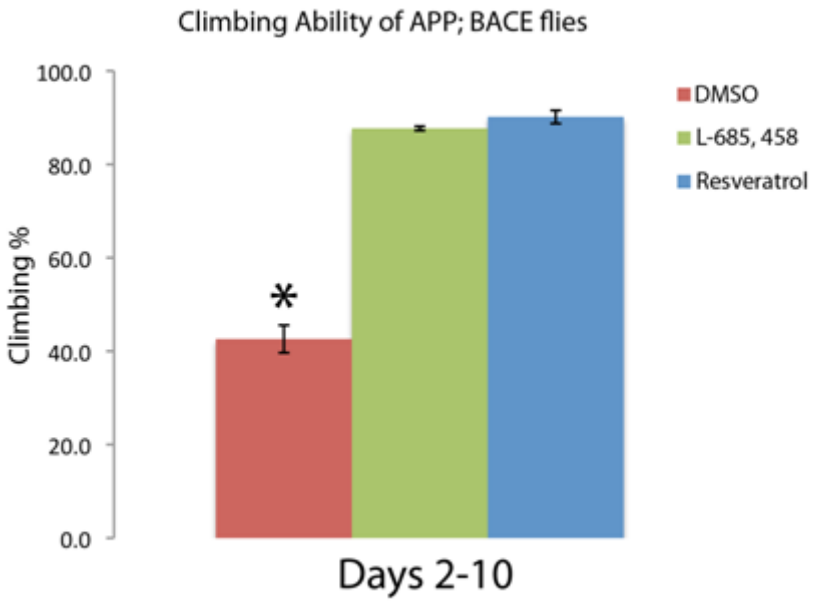


Figure 4-7. Resveratrol rescues climbing ability of AD model flies expressing both APP and BACE



CHAPTER 5: DISCUSSION

Alzheimer's disease (AD) is a major form of dementia in the US, almost exclusively occurring in the population over 65 years old. Age is the most important risk factor for AD. Approximately 90% of all AD cases are referred to as late-onset AD (LOAD), when people develop the disease after 65 years of age. Besides age, many studies have shown that vascular risk factors including diabetes, hypertension, dyslipidemia, and obesity are risk factors for developing AD. Diet plays a strong role these vascular risk factors and on its own is a major environmental factor in organismal aging. In every species tested to date, yeast, roundworm, rodents, and monkeys, dietary caloric restriction confers considerable beneficial health effects. These include extending lifespan and slowing of many age-dependent processes and age-related diseases. Caloric restriction increases lifespan by slowing many aging processes, including normal aging-related changes in the brain. Indeed, the effect of diet in AD has been an area of research that has produced promising results.

Results from a variety of AD models lend support to the idea that diet is a major risk factor for developing AD and caloric restriction might help prevent the development of late onset AD (Wang, Ho et al. 2005; Qin, Chachich et al. 2006; Halagappa, Guo et al. 2007). Caloric restriction has been shown to decrease amyloid plaque deposition in transgenic mouse models of AD (Wang, Ho et al. 2005). Soon after, another group showed that CR resulted in reduced $A\beta_{40}$ and $A\beta_{42}$ peptide levels in the temporal cortex of Squirrel monkeys, compared to (CON/*ad libitum*) fed monkeys. They also reported that this Aβ decrease correlated with an increase in α-secretase active in the brain, which seemed to be due to an increase in SirT1 levels (Qin, Chachich et al. 2006). CR and

intermittent fasting (IF) have also been shown to be neuro-protective against the Aβ and Tau induced decrease in synaptic function in a triple transgenic mouse model of AD (Halagappa, Guo et al. 2007). It has been recently reported that CR attenuates the accumulation of AD-type neuropathology in two cortical brain regions of middle-aged dtg APP/PS1 mice (Mouton, Chachich et al. 2009).

Current AD therapeutics are limited to drugs that treat AD symptoms; so, it is of great interest to identify therapeutics that prevent Aβ-mediated neuronal loss. CR treatment holds considerable potential as a therapeutic strategy for AD. Currently a large body of research is dedicated to understanding the CR mediated changes in aging, lifespan and also the beneficial effects on neurodegenerative diseases like AD. CR results in metabolic changes that induces SIRT1 expression in a wide array of tissues and makes cells more resistant to stress (Brunet, Sweeney et al. 2004). SirT1 has been most commonly associated with the CR mediated changes in organismal longevity in different model organisms (Kaeberlein, McVey et al. 1999; Tissenbaum and Guarente 2001; Hekimi and Guarente 2003; Howitz, Bitterman et al. 2003; Rogina and Helfand 2004; Denu 2005).

I started my work studying the effect of CR on APP metabolism utilizing a previously described *in vitro* model which relies on culturing human cell lines in media supplemented with sera obtained from rats fed *ad libitum* (AL) or caloric restriction (CR) regimens(de Cabo, Furer-Galban et al. 2003; Cohen, Miller et al. 2004). We studied the effect of *in vitro* caloric restriction on APP metabolism utilizing naïve or APP-Gal4 and/or Swedish APP_{695} over-expressing SH-SY5Y and HEK-293 cells, respectively. We observed that *in vitro* CR decreases secreted and intracellular Aβ levels in Swedish

APP_{695} over-expressing HEK-293 cells. We also observed that *in vitro* CR decreases AICD mediated transactivation, but increases sAPPα levels in SY5Y-APP-Gal4 cells. This effect of *in vitro* CR on Aβ and AICD levels could be in part, due to decreased full-length APP levels. Increases in sAPPα levels indicates however, that *in vitro* CR is increasing α-secretase activity in our cell lines, which is in accordance with another study which in a mouse model of AD which has shown that CR also induces the non-amyloidogenic processing of APP (Wang, Ho et al. 2005). So, here we have shown that *in vitro* CR increases sAPPα levels, but decreases AICD levels, indicating that it potentially decreases γ-secretase activity.

In the future we should investigate γ-secretase activity as a result of *in vitro* CR. The previously described model of *in vitro* CR that we used for our study, utilized serum from AL and CR fed F344 (Fisher 344) rats (de Cabo, Furer-Galban et al. 2003). Our results also led us to further study if the effects that we observed on APP metabolism using this model were specific to F344 rat serum. To address that issue, we repeated some of the previous experiments, utilizing a FBN (Fisher Brown Norwegian) AL and CR rat serum keeping all other conditions same. We showed here for that first time that using serum from AL and CR fed FBN rats for this *in vitro* model also decrease AICD mediated transactivation and full-length APP levels. This effect of *in vitro* CR on steady state levels of full-length APP has not been previously reported. This observation may be due simply to the cell types utilized here or it could be a true cellular response to CR. It may be due to CR induced autophagy. Alternatively, this effect on full-length APP could be through a pathway involving genes (like PGC1α) that are induced by metabolic changes as a result of CR. To study the mechanism of the effect of *in vitro* CR

particularly on full-length APP levels, we would conduct different lines of experiments as described later.

Autophagy is a cellular response to limited nutrients to lysosomally degrade non-vital proteins and organelles to produce nutrients ensuring that vital cellular response can continue(Yorimitsu and Klionsky 2005). Lysosomal function(s) decline(s) in older animals. The decline is prevented by CR (Bergamini, Cavallini et al. 2003). In certain organs, like the brain, the proteasomal degradation pathway is more efficiently up regulated during long-term fasting. The proteasome acting together with Ubiquitin and ubiquitin-processing enzymes is responsible for most cytosolic protein degradation under normal nutrient conditions and has a variety of essential functions including protein quality control. It has been shown that during acute nutrient depletion, proteins can be degraded via the proteasomal pathway for generation of amino acids needed for cell maintenance (Takahashi and Goto 2002; Vabulas and Hartl 2005). We intend to investigate the role of both the lysosome and proteosome in the *in vitro* CR mediated decrease in full-length APP levels **(Figure 5-1)**. We would further study if *in vitro* CR also affects APP turnover using pulse-chase experiments. These studies would help us come to a better conclusion about the specific effects of *in vitro* CR on APP metabolism.

As mentioned earlier, caloric restriction extends life span in model organisms, by increasing the levels and activity of Sir2 (Silencing Information Regulator), a member of the conserved Sirtuin family of NAD^+-dependent protein deacetylases (Howitz, Bitterman et al. 2003; Denu 2005). Caloric restriction nutritionally stresses organisms which respond by inducing SIRT1 expression in a wide array of tissues and makes cells more resistant to this and other stressors (Brunet, Sweeney et al. 2004). SirT1 (or Sir2)

expression alone increases stress resistance and extends lifespan of model organisms (Kaeberlein, McVey et al. 1999; Tissenbaum and Guarente 2001; Rogina and Helfand 2004).

In our *in vitro* CR study, induction of SirT1 levels as has been previously observed for this *in vitro* CR experimental paradigm (Cohen, Miller et al. 2004). To determine if the *in vitro* CR mediated effects on APP metabolism are via SirT1, we performed the same line of experiments upon over-expressing SirT1. We specifically wanted to determine if SirT1 over-expression can recapitulate the effects of *in vitro* CR on APP metabolism in our cell culture model. We have shown that SirT1 over-expression recapitulates some, but not all, of the effects of *in vitro* CR on APP metabolism. We specifically observed that over-expressing SirT1 led to a robust and significant decrease in secreted $A\beta_{40}$ levels and AICD levels as observed in case of *in vitro* CR. We also observed that SirT1 over-expression or resveratrol treatment led to an increase in the levels of sAPPα in SY5Y-APP-Gal4 cells, also observed during *in vitro* CR. We also observed that SirT1 over-expression increased C-83 levels in APP Swed293 cells. C83 is co-produced with sAPPα during α-secretase cleavage of APP **(Figure 5-2)**. Both of these observations indicate that SirT1 over-expression, like *in vitro* CR also increases α-secretase activity, which would be further confirmed with future experiments aimed at studying α-secretase activity specifically. This finding is consistent with the increase in α-secretase cleavage of APP observed in transgenic AD models undergoing CR (Qin, Yang et al. 2006) (Wang, Ho et al. 2005). We have previously observed that genetic and pharmacologic induction of α-secretase activity results in increased AICD levels and increased AICD-mediated transactivation (Zhang, Khandelwal et al. 2007). Here,

however, despite the SirT1-mediated induction of α-secretase activity we observe decreased AICD levels and transactivation. This suggests that in these cells SirT1 not only regulates APP metabolism by inducing α-secretase activity but also by negatively regulating AICD production possibly by inhibiting γ-secretase activity. In fact, according to a recent study, there was an increase in the expression of PS1 with age in the hippocampal tissue of SAMP8 (in senescence-accelerated mouse) model of AD (Kumar, Franko et al. 2009). We further intend to study the effect of SirT1 over-expression on α– and γ–secretase activities. In fact, to confirm our finding on the effect of SirT1 on APP metabolism, we would inhibit SirT1, genetically (using dominant negative SirT1 plasmid or knocking down SirT1 using shRNA constructs) and pharmacologically (using inhibitors like Nicotinamide), in our cell lines and monitor the effects on different aspects of APP metabolism. In fact, in an initial experiment we observed that inhibiting SirT1 using a dominant negative plasmid (SirH335Y) increases AICD mediated transactivation compared to the vector only control **(Figure 5-3)**.

SirT1 over-expression, unlike *in vitro* CR, did not affect full-length APP levels in our cell lines, similar to the findings of other groups in different AD models (Qin, Yang et al. 2006). Therefore, SirT1 mediated decreases in Aβ that we observed may be due to increased α-secretase activity. So far, our results indicate that in vitro CR modulates the levels of AICD, Aβ, and α- and possibly γ-secretase activity and that these effects could be mediated through SirT1 which would be validated by future experiments as mentioned above. But the effect of *in vitro* CR on full-length APP levels are not mediated by SirT1. This effect could be through a different pathway that involves genes like PGC1α (**P**PAR**γc**o-activator **1a**lpha) that are induced during metabolic changes induced by CR.

In fact, the composition of F344 AL and CR serum described by de Cabo et al indicated that the CR serum has decreased levels of glucose, insulin and IGF (Insulin-like Growth Factor)-1 **(Figure 5-4)** (de Cabo, Furer-Galban et al. 2003) which can directly and indirectly induce PGC1α (Rodgers, Lerin et al. 2005).

PGC1α is also a substrate for SirT1. Nemoto et al first showed that SirT1 directly interacts with and deacetylates PGC1α , both *in vitro* and *in vivo* (Nemoto, Fergusson et al. 2005). PGC1α is a transcriptional co-activator that senses nutrient availability. It is strongly activated by cAMP and cytokine pathways, important cellular signal that control energy and nutrient homeostasis (Puigserver and Spiegelman 2003). In response to fasting, SIRT1 modulates gluconeogenic/glycolytic pathways in the liver via PGC-1α. It has been reported that once SIRT1 is induced during fasting, it interacts with and deacetylates PGC-1α at specific lysine residues in an NAD^+-dependent manner (Rodgers, Lerin et al. 2005). The decrease in expression levels of PGC1α regulated genes can be reversed by caloric restriction (Corton and Brown-Borg 2005). PGC1α is an important regulator of reactive-oxygen-species (ROS) metabolism. However, RNAi mediated knockdown of PGC1α prevents it from inducing the ROS detoxifying enzymes GPx1 and SOD2 under oxidative stress in the substantia nigra and hippocampal cells of mice (St-Pierre, Drori et al. 2006). In fact it has been shown that resvreatrol rescues AD-like pathological and behavioral changes, and decreases the acetylation levels of PGC1α in p25 transgenic mouse model of AD (Kim, Nguyen et al. 2007). As mentioned earlier, in our cell lines, we have seen that *in vitro* CR decreases secreted Aβ, AICD and full-length APP levels. Again SirT1 and resveratrol decrease secreted Aβ and AICD levels, but increase sAPPα levels. So, we want to extend our studies to further study if the

transcriptional co-activator of PPARγ, PGC1α is associated with these effects. Our experimental approach is illustrated in **Figure 5-5**. I have done some initial experiments where we over-expressed PGC1α along with wild-type SirT1 or a dominant negative SirT1 (SirH335Y) plasmid in SY5Y-APP-Gal4 cells and monitored the effect on AICD mediated transactivation. We found that both PGC1α alone and PGC1α co-expressed with SirT1 resulted in decreased AICD mediated transactivation. This finding was interesting because it indicates that PGC1α might independently modulate APP metabolism. We also observed that in absence of SirT1 (over-expressing dominant negative SirT1), the effect of PGC1α on AICD mediated transactivation is reversed (**Figure 5-6**). This indicates that PGC1α modulates APP metabolism in association with SirT1. Very interestingly, we also observed that PGC1α decreases full-length APP levels independently and in presence of SirT1 (**Figure 5-7**). However, we have previously shown that *in vitro* CR decreases full-length APP levels but SirT1 does not. Therefore, the effect of in vitro metabolism on APP metabolism could be mediated by PGC1α through a pathway independent of SirT1. Based on our preliminary data we think that *in vitro* CR and SirT1 might modulate some of its effects either directly (being deacetylated by SirT1) or indirectly through PGC1α. We intend to study PGC1α as a modulator of APP metabolism in presence or absence of *in vitro* CR conditions. These studies have the potential for identifying and characterizing PGC1α as a novel target for AD therapeutics.

There are a variety of substrates deacetylated by SirT1. Studies suggest that SirT1 controls cellular stress response by regulating FOXO transcription factors. FOXO genes encode family of proteins that function as sensors to the insulin-signaling pathway and as regulators of organismal longevity. In mammalian cells, in response to oxidative stress,

especially H_2O_2 and heat shock, SirT1 deacetylates FOXO3 (Motta, Divecha et al. 2004). SirT1 differentially affects the function of FOXO3 and allows it to transcribe DNA repair target genes and attenuates apoptosis in the presence of stress stimuli (Brunet, Sweeney et al. 2004). In the presence of growth factors and absence of stress, SIRT1 is located in the nucleus and FOXO3 is located in the cytoplasm. In cells subjected to various stressors, including oxidative stress, FOXO3 relocates to the nucleus (Brunet, Sweeney et al. 2004). Brunet *et al.* identified eight phosphorylation sites and five acetylation sites on FOXO3 that are modified by stress stimuli, and these modifications of FOXO3 might be the trigger for the SIRT1 and FOXO3 interaction(Brunet, Sweeney et al. 2004). Gene knock-out studies showed that FOXO3 was more acetylated in mouse embryonic fibroblasts (MEFs) from *SirT1* −/− knockout mice compared with wild-type MEFs, suggesting that SIRT1 influences FOXO3 acetylation *in vivo* (Brunet, Sweeney et al. 2004). *SirT1*$^{-/-}$ embryonic stem cells (ES) transfected with a FOXO DNA binding sites–luciferase construct and FOXO3, showed higher FOXO3 activity compared with wild-type ES cells, suggesting that SIRT1 represses FOXO3 activity in ES cells(Motta, Divecha et al. 2004). It has been shown in a cell-based model that serum deprivation and resvreatrol treatment led to the nuclear translocation of FOXO3 (Stefani, Markus et al. 2007). Interestingly, a recent study has reported that there is a close association between SirT1 mediated effects of CR on non-amyloidogenic APP processing and FOXO transcription factors (Qin, Zhao et al. 2008). According to this study, SirT1 deacetylates and therefore negatively regulates FOXO3a activity in response to CR in Tg2576 mouse model of AD. This results in repression of *Rho-associated protein kinase-1 (ROCK1)* gene expression, and activates non-amyloidogenic α-secretase processing of APP and lowers Aβ

production(Qin, Zhao et al. 2008) **(Figure 5-8)**. Based on these studies it would be interesting to study if the effects of *in vitro* CR and SirT1 over-expresion on APP metabolism we observe are mediated by FOXO3a. A potential experimental approach to address this question is illustrated in **Figure 5-9**.

As I mentioned earlier, it is also possible the *in vitro* CR decreases full-length APP levels through some other mechanism that does not involve any of the above mentioned genes (PGC1α, FOXO3 or SirT1). I have previously described that the F344 CR serum has severely reduced glucose levels. So another possibility is that CR leads to degradation of APP due to low nutrient availability. We studied the effect of glucose and pyruvate availability on APP metabolism in SH-SY5Y cells (described in Chapter 3). We observed that decreasing concentrations of both glucose and pyruvate decrease AICD mediated transactivation and full-length APP levels. However we did not observe any effect of insulin and IGF-1 levels on AICD mediated transactivation in our cell culture model (data not shown). On the other hand, we observed that there was a decrease in AICD mediated transactivation with decreasing levels of fetal bovine serum (FBS) in the media in SY5Y-APP-Gal4 cells **(Figure 5-10)**. We think that these effects are due to an increased APP turnover simply as a result of nutrient deprivation, specifically glucose and pyruvate.

Based on all our *in vitro* studies we have developed a working model **(Figure 5-11)** for describing the effects of *in vitro* CR on APP metabolism. This model will be validated once we finish all our future experiments as described before.

It was important to us to determine if the effects of caloric restriction and SirT1 induction on APP processing that we observed in our *in vitro* model, are in fact

recapitulated in an *in vivo* model system. For this study, we used an established *Drosophila* model, where apoptosis in developing *Drosophila* eyes is dependent on AICD production as previously described using $PS1^{(-/-)}$ flies (Guo, Hong et al. 2003; Gross, Feldman et al. 2008). We further confirm this dependence on γ-secretase activity by treating these flies with a γ-secretase inhibitor (L-685,458) that suppressed the rough eye phenotype. We report for the first time that caloric restriction and/or over-expression of Sir2 (the *Drosophila* homolog of SirT1) suppresses the rough-eye phenotype in the developing eye of AICD reporter flies. Alternatively, the rough eye phenotype was enhanced in reporter flies lacking a functional Sir2. We also observed that the Sir2 agonist resveratrol was able to mimic the effects of Sir2 gain-of-function and caloric restriction, by suppressing the rough eye phenotype in these flies. These *in vivo* studies strongly support our *in vitro* findings. *In vitro* SirT1 activation via over-expression or *in vitro* CR resulted in decreased Aβ and AICD levels which is analogous to the decreased GRIM expression and the resulting decreased rough-eye phenotype *in vivo*.

The *Drosophila* model that we used for the above study expresses the C-terminal fragment of APP produced by BACE cleavage, C99 (Guo, Hong et al. 2003) and therefore does the initial step in regulated intramembraneous proteolysis of APP by β– or α– secretase is not required for AICD production. So our results from experiments utilizing the above mentioned model of Drosophila suggest that CR and Sir2 inhibit γ-secretase activity, which is again in accordance with our *in vitro* findings. Another possible explanation of our findings in these *in vivo* experiments is that the rough eye phenotype is caused by general apoptosis in the fly eyes induced by *Grim* gene expression. L-685,658 mediated rescue of the phenotype does address that issue, but to

further confirm that our observed effects of rough eye phenotype are specifically due to AICD production and not due to general apoptosis, we intend to conduct control experiments in the future.

Other previously described *Drosophila* models of AD rely either on expression the of the toxic $A\beta_{42}$ peptide in the nervous system (Iijima, Liu et al. 2004; Iijima, Chiang et al. 2008), or expression of the human APP and β-secretase (BACE) ubiquitously in all tissues, or in the developing retina (Greeve, Kretzschmar et al. 2004) . Regulated intramembrane proteolysis of APP is a very important aspect that needs to be studied for the development of AD therapeutics. Earlier *in vitro* studies in our laboratory (Zhang, Khandelwal et al. 2007) and other laboratories have identified numerous genes that can modulate APP metabolism. Hence we felt the need to develop a *Drosophila* model of AD that would allow the natural processing of APP by β– and γ–secretase in the central nervous system. To create a more realistic AD model in *Drosophila*, we expressed the human forms of APP and β-secretase (BACE) exclusively in the developing fly nervous system using the Gal4/UAS system (Brand and Perrimon 1993). Expression of full-length APP and BACE in the fly nervous system would allow the amyloidogenic processing of APP by human BACE and fly γ-secretase leading to the production of Aβ peptides. We utilized the *ELAV-Gal4* reagent to limit the expression of these proteins to the fly nervous system only, since the ELAV promoter is specific to the CNS. We have shown that in flies expressing APP only, full-length APP undergoes non-amyloidogenic processing and leads to the production of C83 or CTFα, the α-secretase cleavage product of APP. C99 or CTFβ was observed in flies that express both APP and BACE indicating that amyloidogenic processing of APP by β-secretase is the

predominant processing pathway in these flies. We also show that there is a buiId up of C83 in APP flies and both C83 and C99 in APP;BACE flies treated with the γ-secretase inhibitor L-685,458. We also detected in Aβ peptide in APP;BACE flies and as expected Aβ levels decrease significantly when flies are treated with L-685,458. Interestingly, we also observed that there is a decrease in the levels of full-length APP in APP;BACE flies compared to APP flies, possibly because the abundance BACE in the system is driving APP towards proteolysis. Our findings suggest that we have successfully expressed APP and BACE in our AD model flies and that APP predominantly undergoes amyloidogenic processing in these flies.

APP and BACE were expressed continuously during development to determine if this genotype could produce viable flies with prominent phenotypes that were consistent with nervous system degeneration. We made quite a few interesting observations. First of all, we observed that the number of adult APP and BACE co-expressing flies that eclosed from the crosses were far less compared to the number of APP expressing flies or wildtype out-crossed flies that eclosed from respective crosses. Although we did not quantify this observation, it was definitely a trend that we observed. This led us to think that the expression of APP and BACE are causing a developmental defect that is leading to lethality in the larval and/or pupal stages; hence the smaller number of adult flies emerging from the crosses. In the future we intend to quantify and analyze this observation. Secondly, upon eclosion, we observed two distinct morphological abnormalities in these flies: crumpled wings, consistent with a previous study where only APP was expressed in the flies (Fossgreen, Bruckner et al. 1998) and the presence of melanotic masses on both the abdomen and proboscis of the fly. This feature however has

not been previously described. Formation of these pigmented mass seems to be due to localized buildup of pigment in the presence of tissue damage and/or necrotic tissue in the area. These phenotypes are observed in flies expressing human APP alone, but are greatly enhanced by flies that express both the human APP and BACE proteins, consistent with the idea that the phenotypes are dependent upon the expression of BACE, and may be due to increased accumulation of Aβ toxicity. Both of these phenotypes are strongly suppressed when these flies are treated with L-685,458 strongly suggesting that γ-secretase cleavage of APP and therefore Aβ peptides is somehow responsible for these damages.

Consistent with AD brains, brains from APP;BACE flies showed significant size reductions in a number of brain structures, including the mushroom body, the antennal lobes, and the optic lobes. Structures involved in synaptic function (dendrites and axons) are smaller in overall size in APP;BACE flies compared to controls. Further, this reduction in size is dependent upon both the expression of human BACE, and a functional γ-secretase complex, consistent with the idea that these phenotypes are due to the generation of AICD and Aβ peptides through γ-secretase cleavage of C99.

Our observation of Thioflavin S positive and 6E10 postive puncta in the brains of APP;BACE flies, suggests that the reduction in dendritic and axonal structures observed in these flies may be due to Aβ generation. We further observed that there was a rescue in size of the brain structures in APP;BACE flies that correlated with a significant decrease in Thioflavin S positive puncta and 6E10 positive amyloid plaque distribution in these fly brains on being treated with L-685,458. This further suggests that Aβ peptides or AICD generated through γ-secretase activity are involved in the changes observed in the brains

of these flies. We also observed that APP and BACE over-expressing fly brains were larger in volume when compared to brains of those maintained on vehicle control (DMSO) food **(Figure 5-12)**, consistent with the fact that there is decreased brain size in AD patients due to massive loss of neurons compared to normal individuals.

While these results are consistent with neurodegeneration, our experiments cannot rule out the possibility of developmental defects. To test for deficits in learning and memory in our AD model, we performed the conditioned courtship suppression assay (Siegel and Hall 1979). Interestingly, we observed that our AD model flies expressing APP and BACE are able to successfully perceive and interpret the sensory stimuli in this assay normally, and that they are able to alter their behavior appropriately (learn) in response to training.

Assaying our model flies for immediate recall memorydefects, we observed deficiency in immediate recall memory of this training in APP;BACE flies. However, exposure to L-685,458, showed a clear rescue of this recall defect as compared to parallel sham trained AD flies cultured on this media, indicating that the drug L-685, 458 can suppress the immediate recall memory defect normally associated with AD flies on DMSO.

The AD model flies display defective for normal reflexive CNS behavior represented by significantly reduced climbing ability, consistent with the effects of the neurodegeneration we observe in these flies' brains. Further, both of these behavioral defects can be significantly improved by using L-685, 458, consistent with the idea that these phenotypes also require the presence of Aβ peptides and AICD generated through β- and then γ-secretase cleavage of APP. Therefore we would be able to identify and

characterize regulators of APP proteolysis utilizing this model of AD. In fact, we have further shown that the Sir2/SirT1 agonist Resveratrol, significantly rescued the climbing defects observed in our AD flies further confirming that this would be a good model to screen genetic and pharmacologic regulators of APP metabolism.

Our *Drosophila* AD model is a good model that would be appropriate for studying the effects caloric restriction on APP metabolism. It could extend our understanding of the mechanisms by which CR modulates APP metabolism *in vivo*. We would maintain our control and AD flies that express APP and BACE, in high and low calorie diets as has been done in my previous study on the AICD reporter fly model. We could then conduct all of the above studies on these flies to determine if, and how, CR modulates APP metabolism in our AD model flies. It would also be of great interest to study the effects of Sir2 on our AD model flies. Since we have already shown that resveratrol, the Sir2 agonist rescues climbing behavior in our AD model flies, it would interesting to study different behavioral and neuroanatomical aspects of these flies in conjunction with Sir2 gain and loss-of function. Based on some preliminary results, we would also want to study the effects of PGC1α and FOXO3 on our AD model flies. As mentioned earlier our lab has identified several regulators of APP metabolism utilizing an AICD-based *in vitro* screening (Zhang, Khandelwal et al. 2007). We can validate our *in vitro* findings by studying the effects of these regulators on APP metabolism utilizing our AD model flies.

To summarize, we studied the effects of *in vitro* caloric restriction on APP metabolism in SH-SY5Y and HEK 293 cells. We have shown that *in vitro* CR decreases Aβ, AICD and full-length APP levels in these cell lines without affecting APP

transcription. We have further shown that SirT1 over-expression and resveratrol treatment recapitulates some of the effects of *in vitro* CR on APP metabolism. SirT1 and resveratrol however, do not affect full-length APP levels. In our cell lines, SirT1 and resveratrol reduces secreted Aβ levels by inducing the α-secretase cleavage of APP and also possibly by affecting γ-secretase activity, which need further investigation. Our initial *in vivo* studies utilizing the AICD reporter *Drosophila* model of AD have shown that caloric restriction, Sir2 gain-of-function and resveratrol treatment suppress AD-like rough-eye phenotype in the fly eyes. Finally to study the mechanism of CR, SirT1 mediated effects on APP metabolism *in vivo*, we created a novel *Drosophila* model of AD. We have shown that our model displays neuroanatomical and behavioral features that are characteristic of AD patients.

Figures

Figure 5-1. Experimental design to study the effect of *in vitro* CR on APP metabolism in presence of Proteasomal and Lysosomal inhibitors

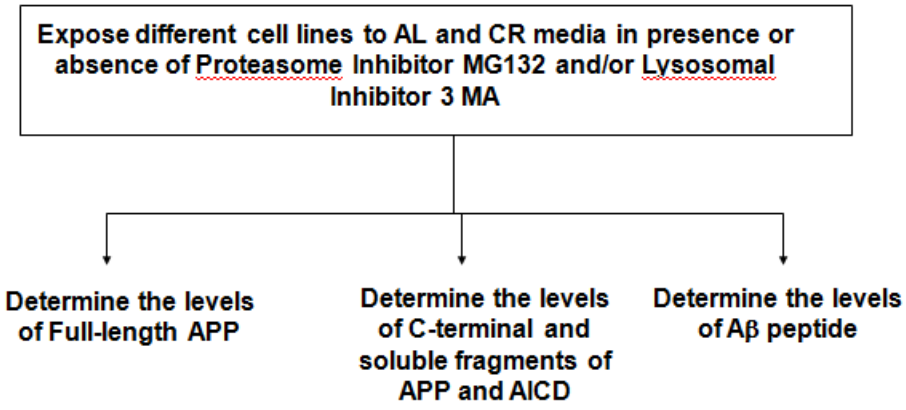


Figure 5-2. SirT1 over-expression increases C83 levels**.** HEK 293 cells stably expressing Swedish APP_{695} were transiently transfected with SirT1 over-expression plasmid and empty vector control. Lysates were used for Western Blot. Equal amount of protein was loaded on gel. β-Actin was used as loading control.

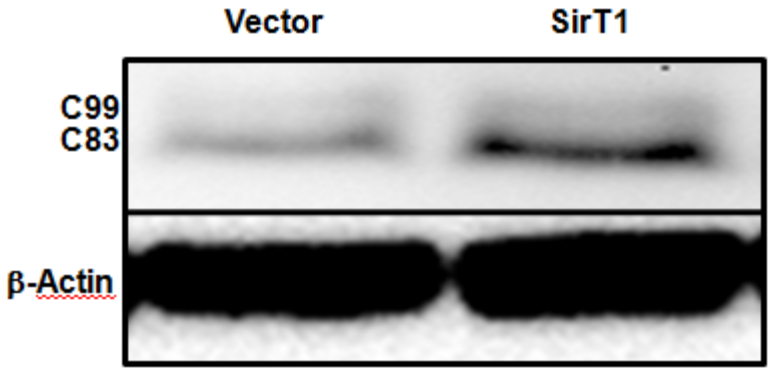


Figure 5-3. SirT1 and dominant negative SirT1 (SirH355Y) modulate AICD mediated luciferase levels. SY5Y APP-Gal4 cells were transiently transfected with Empty vector, SirT1 over-expression and dominant negative SirT1 (SirH355Y) and 1/10th of Renilla luciferase (pRLSV40) plasmids. Lysates were used for Dual luciferase assay. Firefly luciferase counts were normalized to Renilla luciferase counts. Error bars represent standard deviation (n=6). Asterix indicates p-value <0.05.

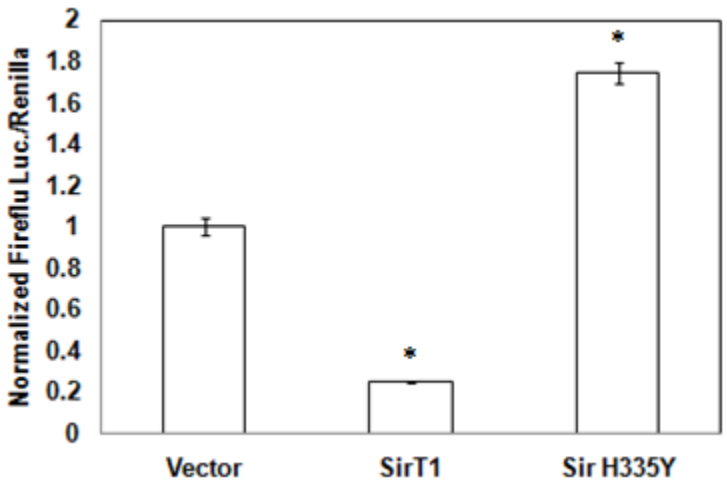


Figure 5-4. Composition of F344 AL and CR Rat serum

Rat AL and CR sera were assayed for the components shown, as described in Section 2 (Anilytics, Gaithersburg, MD)

Parameter	AL	CR	*p*-value
Albumin (g/dl)	4.22 ± 0.34	4.06 ± 0.30	NS
Glucose (mg/dl)	233 ± 60	124 ± 22	**
Total cholesterol (mg/dl)	76 ± 10	79 ± 8	NS
HDL cholesterol (mg/dl)	35 ± 21	34 ± 22	NS
Triglycerides (mg/dl)	189 ± 36	77 ± 27	**
Corticosterone (ng/dl)	833 ± 239	956 ± 295	NS
Free fatty acids (μM)	902 ± 100	1183 ± 128	**
Insulin (ng/dl)	2.5 ± 2	0.31 ± 0.2	**
IGF-1 (ng/dl)	1476 ± 207	1047 ± 33	**

Figure 5-5. Experimental approach to study the involvement of PGC1α in in vitro CR and SirT1 mediated effects on APP metabolism

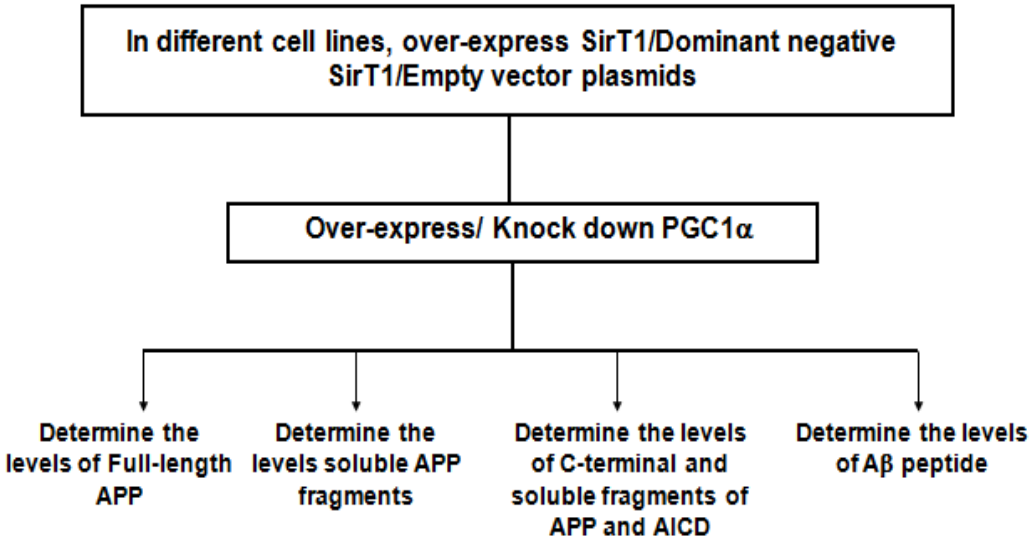


Figure 5-6. PGC1α affects AICD mediated luciferase levels in presence and absence of SirT1.SY5Y APP-Gal4 cells were transiently transfected with Empty vector, PGC1α over-expression, SirT1 over-expression and dominant negative SirT1 (SirH355Y) and 1/10th of Renilla luciferase (pRLSV40) plasmids. Lysates were used for Dual luciferase assay. Firefly luciferase counts were normalized to Renilla luciferase counts. Error bars represent standard deviation (n=6). Asterix indicates p-value <0.05.

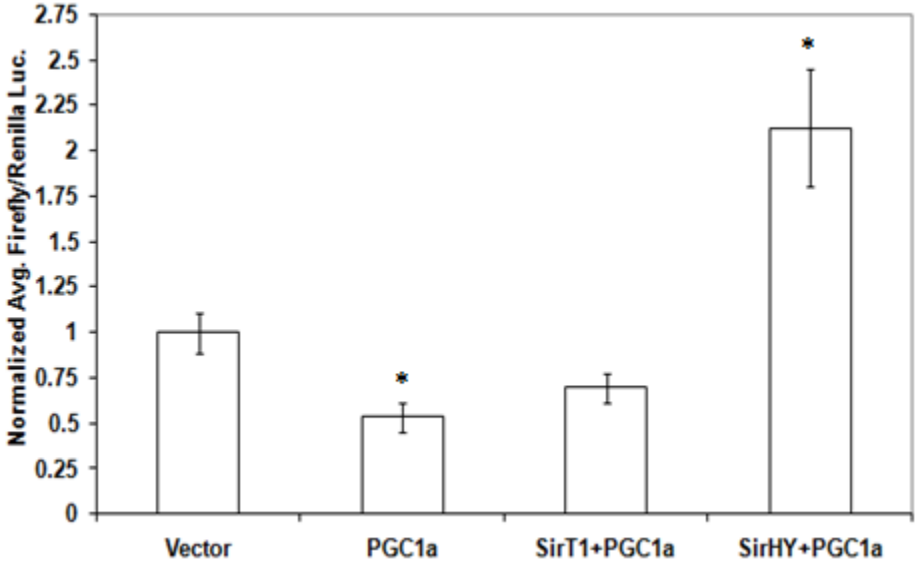


Figure 5-7. PGC1α affects full-length APP levels in presence and absence of SirT1. SY5Y APP-Gal4 cells were transiently transfected with Empty vector, PGC1a over-expression and SirT1 over-expression. Lysates were used for Western Blot. Equal amount of protein was loaded on gel. β-Actin was used as loading control.

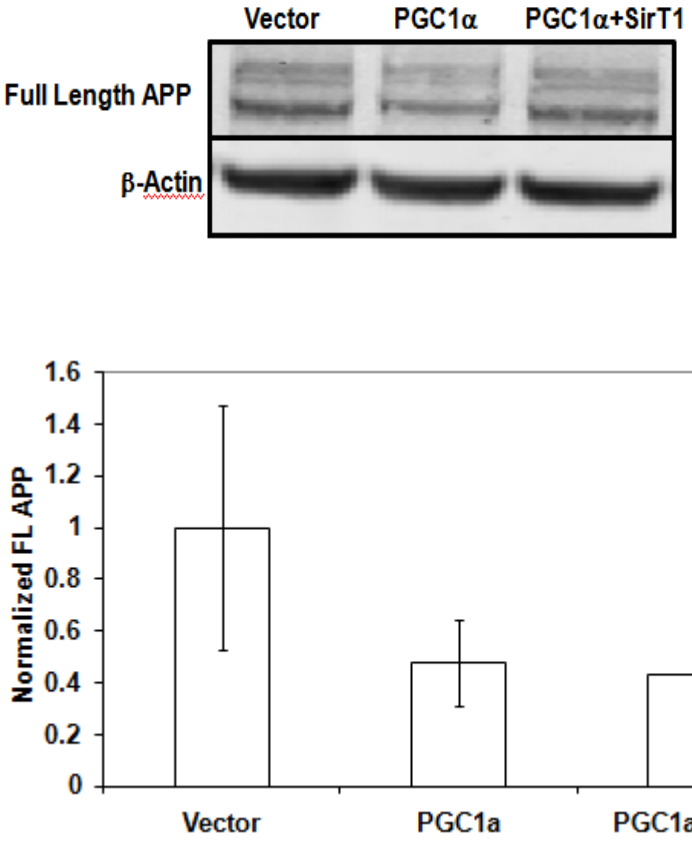
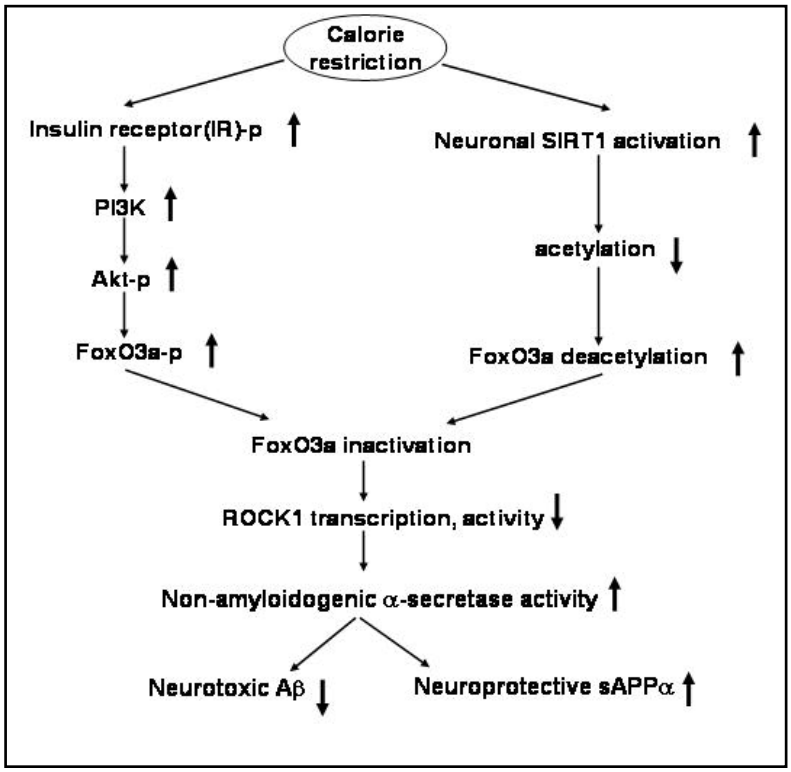


Figure 5-8. Proposed model for FOXO3 involvement in CR mediated effects on APP metabolism



(Qin, Zhao et al. 2008)

Figure 5-9.Experimental approach to study the involvement of FOXO3 in in vitro CR and SirT1 mediated effects on APP metabolism

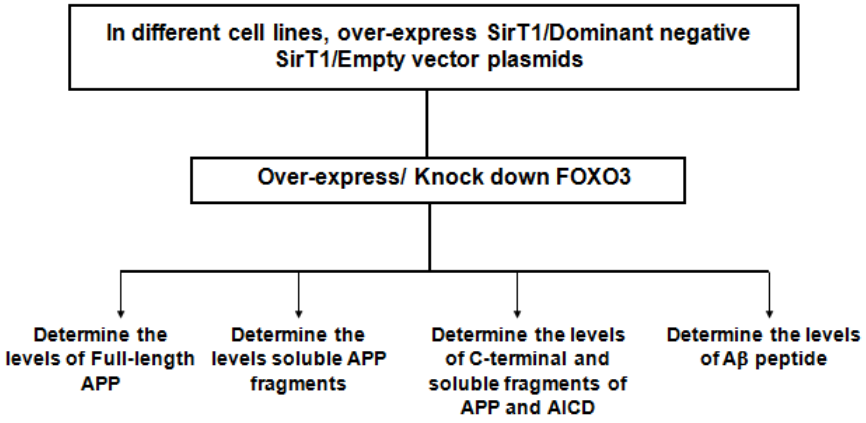


Figure 5-10. Proposed model *in vitro* CR mediated changes on APP metabolism

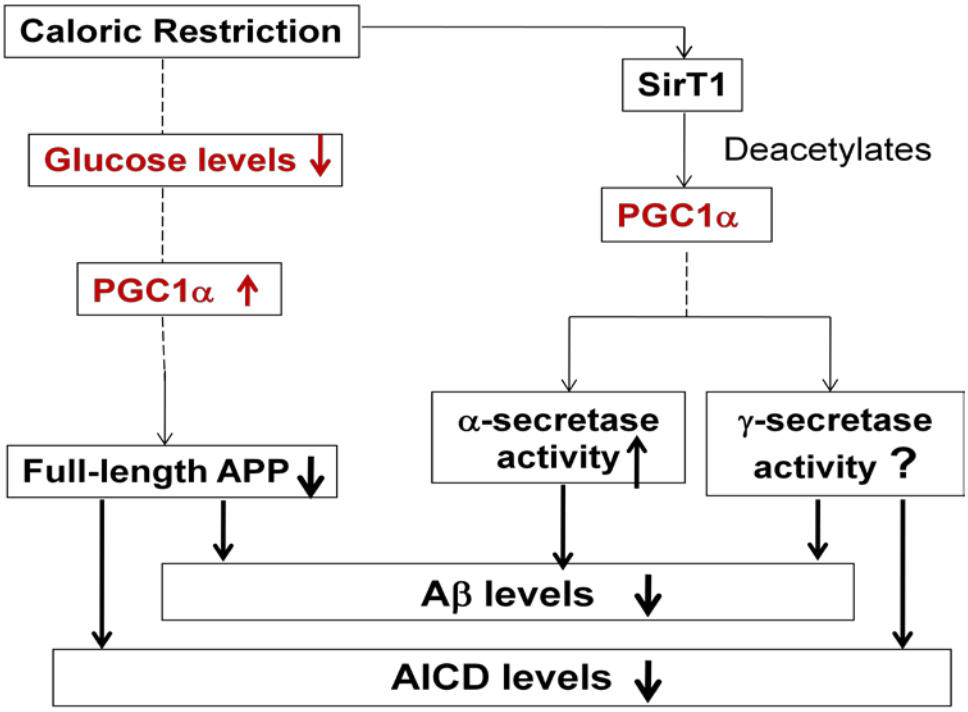


Figure 5-11. Availability of Fetal Bovine Serum (FBS) in media affect AICD mediated luciferase activity.SY5Y APP-gal4 cells were treated with DMEM supplemented with different concentrations of Fetal Bovine serum for 6 hours. Lysates were utilized for Luciferase assay and luciferase counts per second were normalized to total protein concentration (µg/µl). Error bars represent standard deviation (n=3). Asterix indicates p-value <0.05.

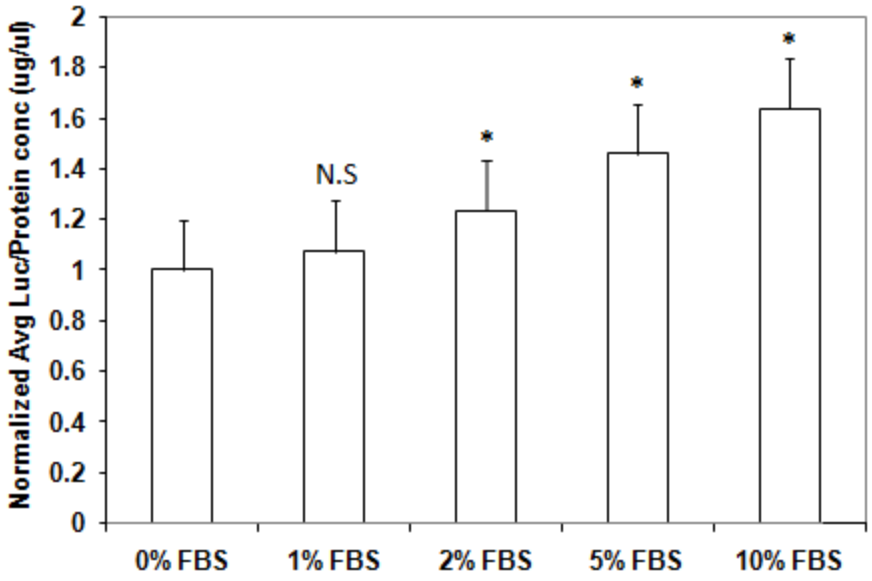
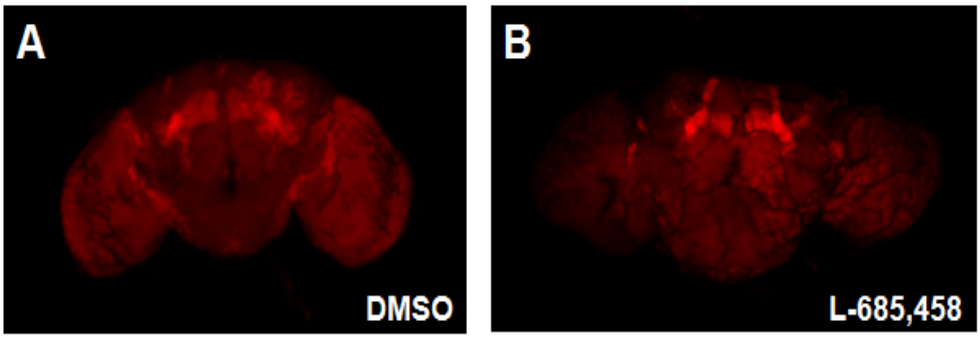
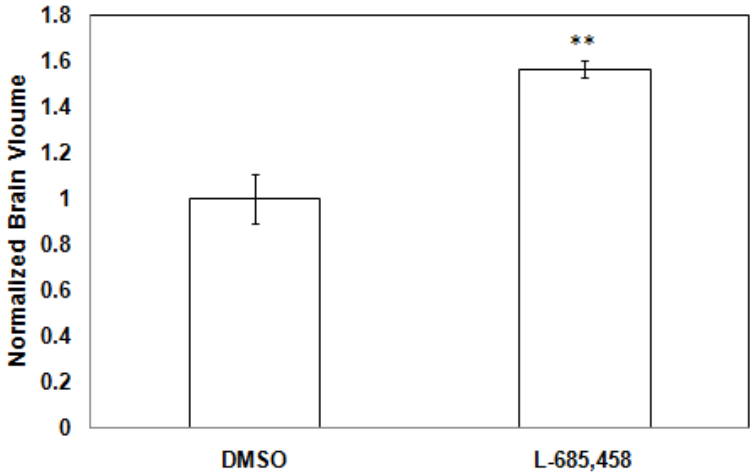


Figure 5-12. Comparison of brain volume of APP and BACE expressing AD model flies maintained in DMSO (vehicle control) and L-685,458





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