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Senior Honors Thesis

A Possible link between Aβ42 production associated with Alzheimer's Disease and SIRT1

Alzheimer's, a neurological disease commonly associated with aging, is believed to be driven by the improper clearance and thereby buildup of the 42 residue Aβ peptide. SIRT1 has a demonstrated role as a regulator of metabolism and the process of aging. Prior *in virto* experiments have shown a protective role of SIRT1 against neurodegenerative diseases like Alzheimer's. Here we show increased levels of SIRT1 results in decreased levels of Aβ42 peptide in the brains of a mouse model for Alzheimer's disease. These results show for the first time *in vivo* an inhibitory role for SIRT1 of Aβ42 production which could have important consequences for the prevention of Alzheimer's disease.

List of Abbreviations Used: AD: Alzheimer's disease, T1 (+/-): SIRT1 hemizygous and thereby low SIRT1 expression levels, Tg: SIRT1 transgenic mice with overexpression of SIRT1, AD mice have the following mutations-APPswe: Swedish mutation of the APP protein, PSEn1dE9: Deletion of exon 9 in the Prensenilin 1 gene, Aβ42: 42 residue long peptide amyloid β-protein

Alzheimer's disease

Alzheimer's disease is a progressive debilitating neurological disorder that currently affects 5.2 million Americans and is the most common cause of dementia in adults. While commonly occurring above the age of 65, this disease is not limited to old age and can occur much earlier. Symptoms of this disease include long term memory loss, cognitive impairment, loss of bodily function and ultimately death. The disease also has an enormous cost to society and caregivers due to the prolonged decline in the health of the individual that necessitates long term care.[1] This combined with the increasing number of elderly Americans makes Alzheimer's disease an intense subject of research.

Currently there is no cure for this disease due in large part to there being no known cause of the disease.[2] There are multiple risk factors for Alzheimer's , with increasing age being the most important. Often risk factors include family history, apolipoprotein E4 status, head injury, depression, hypertension, diabetes, high cholesterol, atrial fibrillation, and low physical and

cognitive activity. There is a genetic contribution to the development of Alzheimer's disease, especially in the case of early-onset, with the risk of first degree relatives getting the disease being 10-40% higher than in unrelated people. However studies have shown only a modest concordance in monozygotic twins which suggests environmental factors play a significant role as well.[1] Regardless of how the disease is initiated, some advancements in research of the pathogenesis of the disease on a molecular level have been made. Much of this work has centered on unlocking the cellular pathways that generate the defining feature of Alzheimer's: insoluble protein deposits in the brain.

The Amyloid Hypothesis

Alzheimer's disease is primarily differentiated from other forms of dementia by the presence of amyloid bodies in the brain. Amyloid is a generic description applied to a heterogeneous class of tissue protein precipitates that have the common feature of beta-pleated sheet secondary structure. Amyloids may be deposited in a general manner throughout the body (systemic amyloids) or confined to a particular organ (e.g. cerebral amyloid, renal amyloid). [3] In Alzheimer's two neuropathological amyloid lesions occur in the meningeal and cerebral vessels, as well as in gray matter: neurofibrillary tangles (NFTs), composed of hyperphosphorylated tau, and extracellular plaques, containing the amyloid β-protein (Aβ).[4] The leading theory of the causes of Alzheimer's is tied to the distinctive plaques of Aβ.

The amyloid hypothesis principally states that the aberrant clearance of Aβ, a product of proteobreakdown in neuronal tissue, results in an initial elevation of soluble Aβ monomers and then oligomers. The subsequent aggregation and deposition of the oligomers into amyloid fibrils leads gradually to widespread synaptic, neuronal, and glial dysfunction accompanied by declining memory and ultimately a multifaceted, profound dementia.[5] The goal would then be to fully elucidate the production pathway of Aβ and find potential targets for therapeutic delay of plaque build up.

Aβ Production and Role in Diseased State

The amyloid β-protein present in plaques is a 40-42 amino acid polypeptide that is the product of the proteolytic cleavage of amyloid β-protein precursor (APP), a large (770 amino acids maximum) type 1 transmembrane glycoprotein widely expressed in mammals.[5] APP has an unknown function in mammals, however it has been implicated in a number of different functions. One study using transgenic knockout mice indicates that APP is closely tied to

synaptic formation and function in young mice.[6] APP is cleaved to Aβ by two sequential cleavages of aspartyl proteases located in the membrane. β- secretase cleaves off the large extracellular domain at Asp672 to generate a 99 residue stub in the membrane. The extracellular receptor region is released into the luminal or extracellular fluid. The stub which remains in the membrane can then be variably cut by γ-secretase into a 38, 40 or 42 residue Aβ peptide with Aβ42 being the most prone to aggregation (Figure 1). Degradation of APP is a normal process, but over production of Aβ42 in certain individuals is sufficient for early onset AD.[7]

A wide body of evidence indirectly connects the pathogenesis of AD to the formation of plaques. The APP protein is located on chromosome 21 in humans and duplications of the APP gene directly relate to increased levels of Aβ protein and increased risk of early onset AD. Trisomies of this chromosome, which occur in Down's syndrome, results in an extremely high rate of early onset AD beginning at age 40. In one case in which a person with Downs had the 21q arm containing the APP gene broken off, the individual lived a dementia free and plaque free life until the age of 78. Additionally, specific mutations in the APP gene near the sites of cleavage or mutations in the proteins of the processing pathway result in increased Aβ levels and an increased risk of early-onset AD. Detractors to the amyloid hypothesis cite the fact that there is a weak correlation in patients with severe dementia and density of fibrilliar amyloid plaques.[7] Countering this a number of molecular studies have shown that soluble Aβ peptides are responsible for impaired synaptic function and are synaptotoxic.[3,6,7]

A major caveat to this research is that a simple cause and effect relationship for the cause of Alzheimer's is highly unlikely. Mutations in APP and in its proteolytic processing are connected to an earlier and heavier plaque load; however the vast majority of cases are seemingly spontaneous and occur only in advanced age. Furthermore finding a target for direct therapeutic intervention thus far has proven very difficult. Inhibitors of γ-secretase and other proteins involved in APP processing have had limited effectiveness. Complicating matters further two forms of Aβ42 exist. One is the insoluble form that has oligomerized to form the plaques. The other is the soluble monomer or dimer of Aβ42. Research has suggested that the soluble form is the actual pathogenic agent and that the plaques are effectively self sequestered from the cells of the nervous system. This could provide an explanation as to why treatments designed to eliminate plaque formations have had limited effectiveness.[7] Taking these facts into consideration an alternate research perspective may be reasonable: investigate Alzheimer's not

as an isolated disease but as a process of the aging nervous system. The expansion of Alzheimer's research into specific regulatory or metabolic pathways affected by aging may lead to viable treatment options.

The Silent Information Regulators: Sirtuins

The connections between aging and Alzheimer's disease are numerous. Alzheimer's disease is the result of a prolonged breakdown in proper proteolytic processing and clearance. The precise mechanisms of aging are unclear but leading theories suggest that it is also a prolonged process in which accumulating damage to the genome and the mitochondria leads to total cellular dysfunction. One possible solution to the problems of aging is to utilize the cell's own putative universal regulator of metabolism and aging: the silent information regulator 2 in yeast and the homologous SIRT1 in mammals.

The Sirtuins are class III histone deacytlases that require NAD^+ as a cofactor and their catalytic domains are highly conserved across all kingdoms. It is theorized the Sirtuins are monitors of cellular energy use and have a wide variety of substrates for which they could be acting as a regulator.[8,9] It is believed that the Sirtuins become active under conditions of cellular stress, such as in caloric restriction, serving to suppress genome instability and alterations in gene expression.[10] Essentially their role may be to maintain the regulatory patterns present in the cell upon replication and to prevent loss of regulation of certain key genes over time.

Caloric restriction (CR) has a number of beneficial effects for cells and organisms. The average and maximum lifespans of a range of organisms — from yeast and nematodes to rodents and monkeys — can be increased by up to 50% simply by reducing their calorie intake compared with what they would eat *ad libitum*. As shown by microarray analysis, CR actually forestalls many progressive changes that occur during aging. CR also reduces the incidence of age-related cancer, cardiovascular disease and immune deficiencies in rodents and in nonhuman primates. [11] Many studies have cemented the connection between CR and the Sirtuins. CR works in yeast and mammalian cells via a *Sir2* mediated mechanism.[10] Small molecule activators of SIRT1 simulate CR in mice fed on a high fat diet.[12] Thus in terms of treating the diseases associated with aging, understanding how to activate the sirtuin genes appears to be a very promising avenue of research.

Prior research has begun unraveling the connections between aging, neurological diseases and sirtuins. In mice strains that develop Alzheimer's plaques very early on (by the first six months), a calorie restriction diet substantially decreases the total accumulation of Aβ plaques.[13] SIRT1 was found to be endogenously up regulated in mice that develop AD, perhaps to counteract the progression of the disease. Furthermore the use of small molecule activators of SIRT1 and virally induced overexpression of SIRT1 result in decreased Aβ production and protection from neurodegradation *in vitro*.[9,13] Presently we sought to further explore this link *in vivo* by examining Aβ peptide production in mice transgenic for SIRT1 and for accelerated amyloid deposition.

A number of mouse models for Alzheimer's disease exist. These strains utilize human mutations in the APP protein and in the proteins required for proteolytic processing that have been shown to lead to early onset AD. The strain for modeling Alzheimer's used in this study utilizes the Swedish APP mutation combined with a deletion in exon 9 of presenilin 1. In this strain called AD mice here the amount of pathogenic Aβ42 is increased while levels of Aβ40 remain constant. Presenilin 1 is connected to Aβ42 production through its requirement for proper γ-Secretase cleavage of APP. AD mice have a greatly accelerated deposition of plaques throughout their lifetime that has been directly correlated to the increased production of Aβ42.[14] An Alzheimer's like state from a histopathologic viewpoint is thereby established in the AD mice. Transgenic mice for SIRT1 have also been generated by the Guarente lab. The T1 (+/-) strain is hemizygous for SIRT1 (+/-) by knocking out one SIRT1 gene. This leads to a decreased expression of SIRT1. In the Tg strain SIRT1 is over expressed by having extra functional copies of the gene inserted (please see Bordone, L. Cohen, D. *et. al.* for further details).[15]

At present progression of amyloid pathology as a function of Aβ42 concentration in mice with varying degrees of SIRT1 expression was studied. AD mice were crossed to the T1 (+/-) strain to examine Aβ42 concentration in the context of low SIRT1 expression. AD mice were also crossed to the Tg strain to examine Aβ42 concentration in the context of high SIRT1 expression. The resulting offspring were sacrificed at the 4 month and 8 month time points. Each mouse's total brain tissue was then measured for Aβ42 concentration via sandwich ELISA specific for Aβ42. Results showed that SIRT1 expression patterns are directly correlated to the reduction in Aβ42 concentration.

Experimental Methods

Breeding of Transgenic Mice

The T1 (+/-) and the Tg strains of mice in a C57/B6J background had been developed previously (Reference 15). The AD mouse strain in a C57/B6J background was purchased from the Jackson Laboratory. Two crosses were performed and checked via genotyping with PCR: AD -T1(+/-) and AD-Tg .

Laboratory animals

Mice were housed in groups of three to five in filter-top cages and were given free access to water and normal chow food. The mice were housed under controlled conditions: temperature (25 ± 1 °C) and light cycle (7:00–19:00 hours). Animals were cared for in accordance to the MIT Committee on Animal Care.

Enzyme-Linked Immunosorbent Assay

At 4 and 8 months of age 3 mice each from AD, AD -T1 +/- and AD -Tg were anesthetized with the general inhalation anesthetic 1-chloro-2,2,2-triflouroehyl difluoromethyl ether and killed by decapitation. This was repeated for WT mice at 4 months, 8 months and 10 months. Brain specimens were harvested and hemi dissected, with one half being frozen for preservation. Half brain samples containing the hippocampus and neocortex were homogenized in 5.0 M guanidine buffer, diluted (1:10) in phosphate-buffered saline containing 0.05% (v/v) Tween-20 and 1 mM Pefabloc protease inhibitors (Roche Biochemicals, Indianapolis, IN) and centrifuged for 20 min at 4°C. A sandwich ELISA for Aβ42 from BioSource, Camarillo, CA was carried out as according to the manufacturer's instructions.

Results

In order to connect SIRT1 expression with amyloid pathology the brains from several types of transgenic mice were measured for Aβ42 concentration via sandwich ELISA (Figure 2). The WT mice showed a constant Aβ42 concentration near 50 pg/ml from 4, 8 and 10 month samples. AD mice showed a more than 100% increase in Aβ42 concentration from 4 to 8 months with a significant increase over the WT. The AD-T1(+/-) cross had an increased concentration of Aβ42 over the AD mouse control at both 4 and 8 months. An opposite effect was seen in the AD-Tg cross where Aβ42 levels showed an almost 100% decrease in Aβ42 concentration at 4 months and a significantly decreased concentration at 8 months when compared to AD mice.

Discussion

The results of this study, for the first time, linked expression levels of SIRT1 with protection against or susceptibility to Aβ42 production *in vivo*. Prior to this the experiments linking the two were limited to tissue culture or AD mice that had been treated with chemical agents that had a proposed effect on SIRT1 expression.[16] The problem with these approaches is that they do not for certain connect SIRT1 and its role in the neurodegeneration of a full organism. My study utilizes the combination of two proven models for AD and SIRT1 expression to more concretely study their connection in a mammalian model. The data is also indirectly relatable to Alzheimer's disease. Aβ42 production has been directly correlated with the development of amyloid pathology associated with Alzheimer's disease.[14] Taken all together it then becomes more reasonable to say that Alzheimer's disease may be prevented in mice by increasing SIRT1 expression. Granted, this study is incomplete at the time of manuscript preparation and while these first steps are important more work needs to be done.

There is a theoretical basis for the connection of SIRT1 and Alzheimer's disease based upon some recent evidence collected in mammalian cells.[10] It was discovered that SIRT1 represses repetitive DNA and a functionally diverse set of genes across the mouse genome. In response to DNA damage, SIRT1 dissociates from these loci and relocalize to DNA breaks to promote repair, resulting in transcriptional changes that parallel those in the aging mouse brain. The loss of silencing over these genes and repetitive DNA sequences may then be a conserved mechanism of aging in eukaryotes. Some of these genes upon activation have been identified as perhaps contributing to Alzheimer's disease. Our data is consistent with this theory.

In young mice Aβ42 production is much lower than in older mice, even in the AD strain. Perhaps as the mice age less SIRT1 is available to silence the genes associated with Alzheimer's because of relocalization. Further in line with this the reduction in Aβ42 production provided by increased SIRT1 expression is much greater in young mice and than in the older mice. If SIRT1 were protecting against Aβ42 buildup by acting on a specific member of the proteolytic pathway then one would assume Aβ42 levels would be consistent over time. Instead its role as an age dependent silencer is more likely with more expression allowing more sites to be covered. Why even over expression could not protect the mice from increasing Aβ42 concentrations over time may be explained by the fact that there may not have been enough over expression. Over time SIRT1 is titrated out over sites spanning the entire genome. Also consistent with this fact is the

drop in the differences between Aβ42 production within SIRT1 expression groups from 4 months to 8 months. It would appear then that as time passes the loss of silencing due to SIRT1 relocalization allows Aβ42 production in AD and AD-Tg mice to catch up to the production levels of AD-T1(+/-). That would serve to imply one or both of the following. That these changes in SIRT1 position are really expansive enough to abrogate SIRT1 inhibitory function even in over expressive mice or that the mutations that generated the AD mouse model are extremely effective and can readily overcome SIRT1suppression. Currently it is impossible to say which it is for certain at this point. But if one were to try to further the point that SIRT1 acts as an age dependent silencer it would be interesting to examine mice under the same conditions at 2 months and 12 months. WT Aβ42 production levels in a 2 month sample of AD-Tg and no detectable difference amongst groups at 12 months would certainly support the theory.

If this area of research continues to produce results consistent with a protective role of SIRT1 against AD it could have important implications for possible treatments. The use of an activator of SIRT1 could halt or reverse the progress of the disease. Resveratrol an activator of SITR1 has already been tied to diabetes prevention. [17,18] The author of this paper has also had an involvement in the development of pharmaceutical activators of SIRT1 through his time at Sirtris Pharmaceuticals. He knows for certain that potent activators of SIRT1 are possible and are currently undergoing clinical trails.

Future directions for research are numerous and varied especially since this study was incomplete at the time this manuscript was written. Western blotting for SIRT1 in all three categories would elucidate some aspects of SIRT1 levels in these mice. Qualitative PCR will be important to establish whether or not SIRT1 expression levels remain constant. Identifying which genes regulated by SIRT1 directly contribute to Aβ42 production will also be important. Additionally, establishing that fact that increased SIRT1 expression actually attenuates symptoms of Alzheimer's disease will really spur on its development as a treatment. Usually demonstrating presentation of the symptoms of Alzheimer's in mice requires a demonstration of reduced cognitive function through behavioral testing. Finally, the precise pathways SIRT1 works through are still highly theoretical, as are the causes of Alzheimer's. The presented connection between the two while extremely interesting, is still very much the subject of debate.

Figures for Walter Barry Thesis

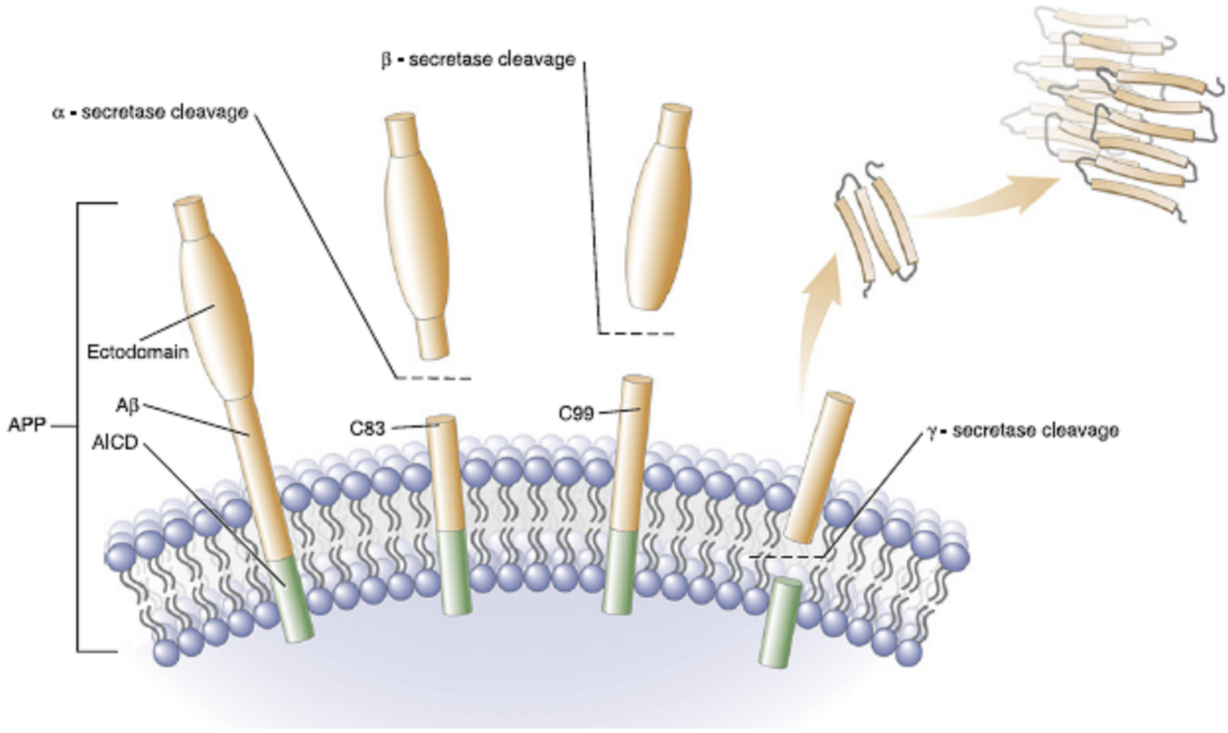

Figure 1: Stepwise Proteolytic Processing of APP by a-, b-, and g-Secretases

Major cleavage sites of the three secretases in APP are indicated by dashed horizontal lines. The TMD is depicted as a cylinder. The Ab-domain is depicted in orange, and the AICD in green. Figure is not drawn to scale. Ab represents amyloid b peptide; AICD, APP intracellular domain; APP, amyloid precursor protein; C83, 83-residue APP C-terminal fragment; C99, 99-residue APP C-terminal fragment. [Taken from reference 5]

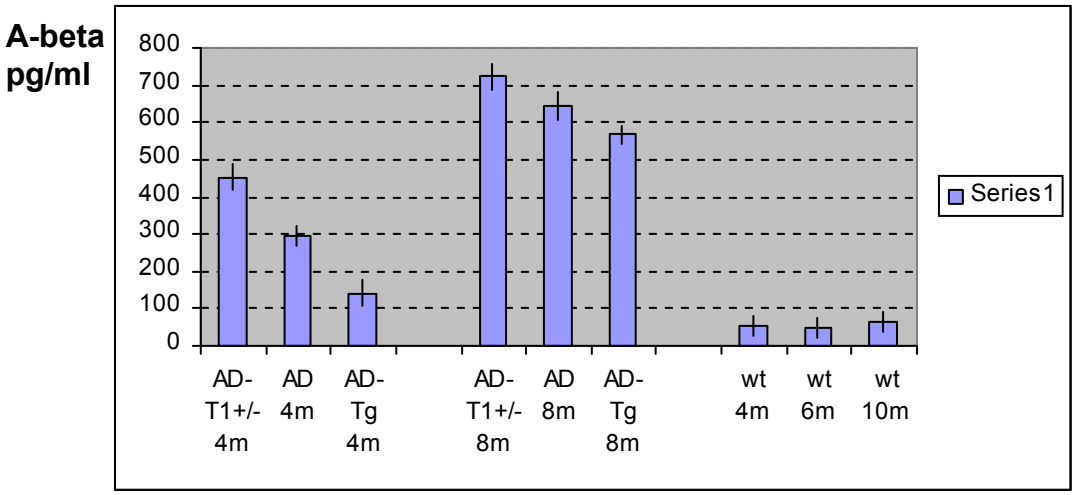

Figure 2 Concentration of Aβ42 peptide from mice cerebral tissue as measured by ELISA

SIRT1 expression has an inhibitory effect on Aβ42 production. Shown are AD mice and the two crosses for SIRT1 expression made with these mice. Error bars listed as ± 1 SD.

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