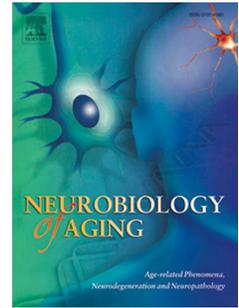


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Sex differences in metabolic aging of the brain: insights into female susceptibility to Alzheimer's disease

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Abstract

Despite recent advances in the understanding of clinical aspects of sex differences in Alzheimer's disease (AD), the underlying mechanisms, for instance, how sex modifies AD risk and why the female brain is more susceptible to AD, are not clear. The purpose of this study is to elucidate sex disparities in brain aging profiles focusing on two major areas – energy and amyloid metabolism – that are most significantly affected in preclinical development of AD. Total RNA isolated from hippocampal tissues of both female and male 129/C57BL/6 mice at ages of 6, 9, 12, or 15 months were comparatively analyzed by custom-designed Taqman low-density arrays (TLDA) for qRT-PCR detection of a total of 182 genes involved in a broad spectrum of biological processes modulating energy production and amyloid homeostasis. Gene expression profiles revealed substantial differences in the trajectory of aging changes between female and male brains. In female brains, 44.2% of genes were significantly changed from 6 months to 9 months and two thirds showed downregulation. In contrast, in male brains, only 5.4% of genes were significantly altered at this age transition. Subsequent changes in female brains were at a much smaller magnitude, including 10.9% from 9 months to 12 months and 6.1% from 12 months to 15 months. In male brains, most changes occurred from 12 months to 15 months and the majority were upregulated. Further, gene network analysis revealed that clusterin appeared to serve as a link between the overall decreased bioenergetic metabolism and increased amyloid dyshomeostasis associated with the earliest transition in female brains. Together, results from this study indicate that: 1) female and male brains follow profoundly dissimilar trajectories as they age; 2) female brains undergo age-related changes much earlier than male brains; 3) early changes in female brains signal the onset of a hypometabolic phenotype at risk for AD. These findings provide a mechanistic rationale for female susceptibility to AD and suggest a potential window of opportunity for AD prevention and risk reduction in women.

Keywords:

Alzheimer's disease; sex differences; metabolic aging; energy metabolism; amyloid metabolism, prevention, risk reduction

1. Introduction

As the leading cause of dementia and rated as the most feared human disease by the American public, Alzheimer's disease (AD) currently affects approximately 35 million people worldwide, including 5.1 million Americans (Thies and Bleiler, 2013). These numbers are predicted to triple by 2050, with one new case of AD expected to develop every 33 seconds, or nearly a million new cases per year (Thies and Bleiler, 2013). There is no cure currently available, and no success has been found from over 100 human trials conducted over the last decade in an attempt to find an effective treatment for mid to late-stage AD (McBride, September 14, 2012; Schnabel, July 8, 2013). These unprecedented challenges underscore the significance and priority for increased research efforts aimed at defining the risk factors and the underlying mechanisms that would allow institution of AD prevention and early intervention while a treatment continues to be sought (Mullard, 2012; Rice, January 25, 2014).

AD affects women and men differently on many levels (Carter et al., 2012; Regitz-Zagrosek and Seeland, 2012). Of the current AD cases, nearly two-thirds are women (Brookmeyer et al., 2011). After age 65, the lifetime risk of AD is 1 in 6 for women (16.7%), whereas it is 1 in 11 for men (9.1%) (Thies and Bleiler, 2013). In addition, sex has been demonstrated to play a major role in the pathogenesis, progression, and clinical manifestation of AD. For instance, depression is associated with a 2-fold increased risk for AD in women but not in men; in contrast, stroke is associated with a 3-fold increased risk for AD in men but not in women (Artero et al., 2008). Women with AD tend to exhibit a broader spectrum of dementia-related behavioral symptoms and experience greater cognitive deterioration than men in the progression of the disease (Barnes et al., 2005; Chapman et al., 2011; Hall et al., 2012; Irvine et al., 2012; Schmidt et al., 2008). Moreover, several studies presented at the recent Alzheimer's Association International Conference provide new evidence supporting the long-held belief that women's brains are more vulnerable than men's brains to AD (Frantz, 2015; Hamilton, 2015).

Increasing evidence indicates that sex also modulates the impact of genetic factors in the etiology of AD. Cognitively normal individuals with a maternal family history of AD were found to express greater phenotypic changes in AD-vulnerable brain regions suggesting a higher risk for developing AD as compared to those with a paternal history or no family history (Berti et al., 2011; Honea et al., 2011; Mosconi et al., 2010). Furthermore, some genetic variants appear to interact with sex to modify the risk for AD. As an example, the $\epsilon 4$ allele of the apolipoprotein E gene (APOE4), the strongest genetic risk factor for late-onset AD, has been associated with a far more pronounced risk for AD in women than in men (Altmann et al., 2014; Bretsky et al., 1999; Farrer et al., 1997; Mortensen and Høgh, 2001; Payami et al., 1996; Ungar et al., 2014). In contrast, the $\epsilon 2$ allele of the APOE gene (APOE2), which is considered as a neuroprotective variant, has been shown to confer a greater protection against AD in men than in women (Altmann, et al., 2014; Johnson et al., 1998; Ungar, et al., 2014).

Despite recent advances in the understanding of clinical aspects of sex differences in AD, the underlying mechanisms, for instance, how sex modifies AD risk and why the female brain is more susceptible to AD, are not clear. In this study, using custom-designed Taqman low-density arrays (TLDA) and Ingenuity pathway analysis (IPA) computing tools, we sought to determine sex disparities in brain aging profiles focusing on two major areas – energy and amyloid metabolism – that are most significantly affected in preclinical development of AD. The data revealed substantial differences in the overall trajectory of aging changes between female and male brains. Early changes indicative of the onset of a hypometabolic phenotype in female brains could serve as an important mechanistic rationale for female susceptibility to AD; and timely intervention at this transition could potentially halt the progression of metabolic deficits and ultimately reduce the risk of developing AD in women.

2. Materials and Methods

2.1. Animals

The use of animals was approved by the Institutional Animal Care and Use Committee at the University of Southern California and followed NIH guidelines for the care and use of laboratory animals. Animals were bred and maintained under controlled conditions of temperature (22 °C), humidity, and light (14 h light, 10 h dark) with water and food available *ad libitum*. Both females and males, 4 age groups (5-6, 9-10, 12-13, and 15-16 months), 5 mice per sex × age group, a total of 40 129/C57BL/6 mice were used in this study. Hippocampal tissues collected from one hemisphere of mice brains were used for TLDA gene expression analysis; tissues collected from the other hemisphere were used for Western blot protein expression analysis. The gene network was generated using the IPA bioinformatics program. A detailed description of the methods for both TLDA and IPA studies can be found in our recent report (Zhao et al., 2012).

2.2. TLDA gene expression profiling

TLDAs with two custom designs in 96-format (91 target genes and 5 candidate control genes were included on each design) were manufactured at Applied Biosystems (Foster City, CA). Mouse hippocampal RNA were isolated using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). RNA quantity and quality were analyzed using the Experion RNA StdSens Analysis Kit on an Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). RNA to cDNA synthesis was prepared using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) on a MyCycler Thermal Cycler (Bio-Rad). Taqman qRT-PCR reactions were performed on 50 ng cDNA samples mixed with the TaqMan Universal PCR Master Mix 2X (Applied Biosystems), under the thermal cycling conditions:

stage 1: AmpErase UNG activation at 50 °C / 2 min / 100% ramp; stage 2: AmpliTaq gold DNA polymerase activation at 94.5 °C / 10 min / 100% ramp; stage 3: melt at 97 °C / 30 sec / 50% ramp, followed by anneal/extend at 59.7 °C / 1 min / 100% ramp, for 40 cycles. Fluorescence was detected on an ABI 7900HT Fast Real-Time PCR System equipped with the Sequence Detection System Software Version 2.3 (Applied Biosystems). Data were analyzed using the RQ Manager Version 1.2 and DataAssist Version 2.0 (Applied Biosystems). Gene expression fold changes relative to the comparison group were calculated by the comparative Ct ($\Delta\Delta C_t$) method, with Ct denoting threshold cycle (Livak and Schmittgen, 2008). Selection of the endogenous control gene for normalization was based on the control stability measure (M), which indicates the expression stability of control genes on the basis of non-normalized expression levels. Four samples per sex \times age group were included in the analysis. For each sample, ΔC_t was calculated as the difference in Ct of the target gene and the endogenous control gene. For each group, mean $2^{-\Delta C_t}$ was calculated as the geometric mean of $2^{-\Delta C_t}$ of the 4 samples in the group. Fold change was then calculated as $\text{mean } 2^{-\Delta C_t (\text{target group})} / \text{mean } 2^{-\Delta C_t (\text{comparison group})}$. Fold change values greater than zero indicate a positive expression or up-regulation relative to the comparison group. Fold change values less than zero indicate a negative expression or down-regulation relative to the comparison group. The $2^{-\Delta C_t}$ values for each target gene were statistically compared between the target and comparison group using Student's t-test. The statistical significance was indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; P-values were unadjusted by multiple testing corrections, thus it is possible that some of the results may not reach significance when a correction is applied. The volcano plot displays fold changes (X-axis) and P values (Y-axis) enabling identification of genes with both large and small expression changes that are statistically significant. The heat map displays results of unsupervised hierarchical clustering. Distances between assays/samples are calculated based on the ΔC_t values using Pearson's Correlation. The ΔC_t value of the middle/median expression level is set such that red indicates an increase with a ΔC_t value below the middle level, and green indicates a decrease with a ΔC_t value

above the middle level. In the assay centric map, for each assay, the middle expression level is set as the median of all of the ΔC_t values from all samples for that assay. Data points for a given assay can only be compared relative to other data points for that assay.

2.3. IPA gene network profiling

IPA (Ingenuity Systems, www.ingenuity.com), which leverages the Ingenuity Knowledge Base, the largest database housing biological and chemical relationships extracted from the scientific literature, provides a bioinformatics computing tool to interpret the experimental gene expression data in the context of biological processes, pathways, and molecular networks. The IPA network analysis identified biological connectivity among molecules in the gene expression dataset that were significantly up- or down-regulated (these molecules are called “network eligible molecules” or “focus molecules” that serve as “seeds” for generating networks) and their interactions with other molecules present in the Ingenuity Knowledge Base. Network eligible molecules were combined into networks that maximized their specific connectivity. Additional molecules from the Ingenuity Knowledge Base (these molecules are called “interacting molecules”) were used to specifically connect two or more smaller networks to merge them into a larger one. A network was composed of direct (requiring direct physical contact between two molecules) and indirect (mediated by intermediate factor(s)) interactions among focus molecules and interacting molecules. Generated networks were ranked by the network score according to their degree of relevance to the network eligible molecules from the dataset. The network score was calculated with Fisher’s exact test, taking into account the number of network eligible molecules in the network and the size of the network, as well as the total number of network eligible molecules analyzed and the total number of molecules in the Ingenuity Knowledge Base that were included in the network.

2.4. Western blot protein expression analyses

Total protein samples were extracted from mouse hippocampus as previously described (Zhao et al., 2009). 20-40 µg of protein samples were loaded per lane and separated by electrophoresis on a 10-12% SDS-PAGE. Proteins were then transferred to 0.2 µm PVDF membranes and probed with primary antibodies against insulin-like growth factor 1 (IGF1; 1:1000, Abcam, Cambridge, MA), clusterin (CLU; 1:1000, Santa Cruz Biotechnology, Dallas, TX), estrogen receptor α (ER α ; 1:1000, Santa Cruz Biotechnology), estrogen receptor β (ER β ; 1:500, Santa Cruz Biotechnology), G protein-coupled receptor 30 (GPR30; 1:750, Santa Cruz Technology), progesterone receptors (PRs; 1:1000, Santa Cruz Technology), and progesterone receptor membrane component 1 (PGRMC1; 1:1000, Cell Signaling Technology, Danvers, MA), at 4°C overnight, and then with HRP-conjugated secondary antibodies (1:3000~10000, Thermo Scientific Piece, Rockford, IL). β -tubulin (1: 5000, Thermo Scientific Piece) or β -actin (1:3000, Thermo Scientific Piece) was used as the loading control. Bands were visualized by chemiluminescence with an ECL detection kit (Bio-Rad). Relative intensities of the immunoreactive bands were captured by a C-Digit Chemiluminescence Western Blot Scanner and quantitated by Image Studio Version 4.0 (LI-COR, Lincoln, NE). Statistically significant differences between groups (N = 5) were determined by one-way analyses of variance followed by Student-Newman-Keuls pairwise multiple comparison post hoc tests. The statistical significance was indicated by * P < 0.05, ** P < 0.01 and *** P < 0.001.

Results

Total RNA isolated from hippocampal tissues of both female and male 129/C57BL/6 mice at ages of 6, 9, 12, or 15 months were comparatively analyzed by custom-designed TLDA for qRT-PCR detection of a focused set of 182 genes involved in a broad spectrum of biological processes modulating energy production and amyloid homeostasis. Among 5 candidate control genes (18S, Actb, Gapdh, Hprt1, and Ipo8), Gapdh exhibited the most stable expression (*M*) across all samples and thus was used as the control gene for normalization in the following data analysis. Of the 182 target genes assayed, 17 genes that showed low expression with Ct values > 35 were excluded from the dataset (Tables S1 & S2); 165 genes with Ct values < 35 are included in Figs. 1, 2A, 2B, and Table S3.

Gene expression profiles revealed substantial disparities in the overall trajectory of aging changes between female and male brains; and female brains exhibited a much earlier onset of changes compared to male brains ((Figs. 1, 2A, 2B, Table S3). Specifically, in female brains, most changes (73 out of 165 genes; 44.2%; 23 increase and 53 decrease) occurred between the ages of 6 months and 9 months (Figs. 1, 2A, 2B, 3). Among these, 37 genes involved in energy metabolism exhibited a fold change > 1.4-fold, and the majority were downregulation (32 decrease, 5 increase), including: 1) four genes involved in insulin/insulin-like growth factor (IGF) signaling: Foxo1 (FC = -2.36, P = 0.012), Igf1 (FC = -1.45, P = 0.031), Irs2 (FC = -1.83, P = 0.0025), and Prkaa1 (FC = -1.72, P = 0.026); 2) two genes involved in glycolysis: Ldhd (FC = -2.01, P = 0.000001), Ldhb (FC = -2.01, P = 0.0024); 3) four genes involved in pyruvate dehydrogenase (PDH) complex and tricarboxylic acid (TCA) cycle: Pdk2 (FC = -1.70, P = 0.020), Pdk3 (FC = -1.71, P = 0.0052), Sdha (FC = -1.44, P = 0.027), and Sdhb (FC = -1.55, P = 0.0018); 4) four genes involved in electron transport chain (ETC) and oxidative phosphorylation (OXPHOS): Ndufs4 (FC = -1.88, P = 0.0011), Ndufs8 (FC = -1.75, P = 0.00073), Uqcrc2 (FC = -1.42, P = 0.0077), and Uqcrcs1 (FC = -1.74, P = 0.00087); 5) seven genes involved in reduction and oxidation (Redox): Glrx

(FC = -2.04, P = 0.0044), Glrx2 (FC = -2.07, P = 0.00053), Gpx1 (FC = -1.96, P = 0.0018), Gpx4 (FC = 1.47, P = 0.037), Prdx3 (FC = -1.47, P = 0.000023), Sod1 (FC = 2.09, P = 0.00077), and Sod2 (FC = -1.43, P = 0.000077); 6) nine genes involved in transport: Hspd1 (FC = -2.30, P = 0.00013), Slc25a14 (FC = -1.60, P = 0.0017), Slc25a27 (FC = 1.68, P = 0.021), Slc25a4 (FC = -2.21, P = 0.0000003), Slc2a1 (FC = 1.78, P = 0.020), Slc2a3 (FC = 1.44, P = 0.022), Timm23 (FC = -1.99, P = 0.0015), Timm20 (FC = -1.83, P = 0.0037), Vdac2 (FC = -1.48, P = 0.030); 7) one gene involved in apoptosis: Bcl2 (FC = -1.58, P = 0.039); 8) nine genes involved in lipid/ketone metabolism and cholesterol trafficking: Acaa1a (FC = -2.57, P = 0.000011), Acaa2 (FC = -1.70, P = 0.0031), Acadl (FC = -2.19, P = 0.0097), Acadm (FC = -1.49, P = 0.027), Acadvl (FC = 1.60, P = 0.045), Acat1 (FC = -1.58, P = 0.017), Cpt2 (FC = -1.59, P = 0.00090), Decr1 (FC = -1.76, P = 0.0045), and Rarb (FC = -1.69, P = 0.0022). No genes involved in mitochondrial biogenesis and dynamics exhibited a fold change > 1.4-fold at the transition from 6 months to 9 months in female brains.

In regards to genes involved in amyloid metabolism, 10 genes exhibited a fold change > 1.4-fold at 9 months relative to 6 months in female brains, including six that increased in expression: Apbb1 (FC = 1.53, P = 0.032), Apbb3 (FC = 1.91, P = 0.035), Bace1 (FC = 1.57, P = 0.025), Ctsb (FC = 1.50, P = 0.036), Ncstn (FC = 1.44, P = 0.0057), and Psen1 (FC = 1.49, P = 0.022), and four that decreased in expression: Apba2 (FC = -1.41, P = 0.038), App (FC = -1.48, P = 0.0061), Nae1 (FC = -2.07, P = 0.0087), and Psenen (FC = -2.09, P = 0.00063). Additionally, two genes that are recently identified as genetic risk factors for late-onset AD exhibited a decreased expression > 1.4-fold, including Clu (FC = -1.84, P = 0.0016) and Picalm (FC = -1.43, P = 0.0065).

Compared to the changes in female brains that occurred at the transition from 6 months to 9 months, there were not only a much smaller number of genes but also the majority of them were altered at a much smaller magnitude at transitions from 9 months to 12 months and from 12 months to 15 months. A total

of 18 genes (10.9% of the total number of genes analyzed) were significantly changed from 9 months to 12 months, and only three of them exhibited a fold change > 1.4-fold, including: *Foxo1* (FC = -1.40, P = 0.026), *Gpx3* (FC = 1.44, P = 0.030), and *Pcdh11x* (FC = -2.04, P = 0.024). Similarly, a total of 10 genes (6.1% of the total number of genes analyzed) were significantly changed from 12 months to 15 months, and only one of them exhibited a fold change > 1.4-fold, *Cyp27a1* (FC = 1.85, P = 0.029).

Male brains exhibited two major differences compared to female brains. First, male brains underwent substantially less changes between the ages of 6 months and 9 months; nine genes were significantly changed, 2 increased and 7 decreased, representing 5.4% of the total number of genes analyzed. In addition, the magnitude of changes in male brains was much smaller than those that occurred in female brains; all nine genes exhibited a fold change < 1.3-fold. Similarly, a total of nine genes exhibited a significant change from 9 months to 12 months, and they all had a magnitude of change < 1.3-fold. The second major difference between male and female brains was detected between the ages of 12 months and 15 months. During this age period, female brains exhibited the fewest changes in both the number of genes and the overall magnitude, whereas male brains underwent the most changes involving a total of 45 genes, representing 27.3% of the total number of genes examined. Among them, the majority presented an increase in expression (35 increase, 10 decrease), and eight genes exhibited a fold change > 1.4-fold, including: five increases in expression: *Igf1* (FC = 1.63, P = 0.00046), *Prdx3* (FC = 1.41, P = 0.000024), *Mfn1* (FC = 1.54, P = 0.0055), *Bcl2* (FC = 6.29, P < 0.0000001), and *Bace2* (FC = 1.94, P = 0.038), and three decreases in expression: *Bcl2l1* (FC = -5.86, P < 0.0000001), *Nr1h3* (FC = -1.91, P = 0.016), and *Apba3* (FC = -3.52, P = 0.0021) (Figs. 1 and Table S3).

The graph in Fig. 4 displays the IPA network (with a network score of 85), with the most relevance to gene expression changes occurring at the transition from 6 months to 9 months in female brains. Within this large network that includes 40 focus molecules (referring to significantly changed genes included in

the dataset), two sub-networks were revealed. One sub-network was largely populated with bioenergetic genes and many of them were decreased, in which *Igf1* appeared to serve as a central node. The other sub-network was populated primarily with genes involved in amyloid production and many of them were increased. *Clu* appeared to serve as a linker between the two sub-networks.

Changes in the expression of *Igf1* and *Clu* at the transition from 6 months to 9 months in female brains were further confirmed by western blot analyses. Consistent with the gene expression data, the protein levels of both *Igf1* (FC = 0.73 9 months vs 6 months; $P = 0.029$) and *Clu* (FC = 0.68 9 months vs 6 months; $P = 0.042$) were significantly decreased at 9 months compared to 6 months (Fig. 5). Among the six major estrogen and progesterone receptors analyzed, only $ER\beta$ showed a significant change between 6 months and 9 months (FC = 0.70 9 months vs 6 months; $P = 0.047$) (Table 1 and Fig. S1).

Discussion

AD disproportionately affects women more than men. Women have a greater lifetime risk of developing AD and constitute two thirds of the current AD population (Brookmeyer, et al., 2011; Thies and Bleiler, 2013). The prevailing view has been that women's susceptibility to AD is due to the fact that they live longer and hence have a higher age-associated risk for AD, which is true to a minimal extent. Statistics show that the living age difference between men and women is not as large as one might think. Currently, the average life expectancy worldwide for all people is 71 years, 69 years for men and 73 years for women, a difference of four years (www.statista.com, 2015), whereas on average people with AD can live eight to 10 years from the diagnosis (<http://www.alzheimers.org.uk>, 2015). Results from a meta-analysis of seven sex-specific studies concluded that women were 1.5 times more likely to develop AD than age-matched men (Gao et al., 1998), which is consistent with the data derived from the Cache County study that showed a clear higher incidence of AD in women (Zandi et al., 2002). These findings suggest that age does not solely account for the sex disparity in the prevalence of AD, and there must be other factors that play an even greater role in predisposing females to a higher level of risk for AD. Understanding the biological bases underlying such sex differences in AD could potentially lead to new directions for preventing or reducing the risk of developing the disease, especially in the high-risk population of older women.

In this study, using custom-designed TLDA gene array profiling and IPA bioinformatics computing tools, we analyzed the expression aging profile and interactive network of a focused set of genes in the hippocampus of both female and male mice. The target genes were selected based on their involvement in energy and amyloid metabolism, two major areas that are most significantly affected in preclinical development of AD (Brinton, 2009; Mosconi et al., 2006; Swerdlow et al., 2013; Yao et al., 2009). The results revealed substantial disparities in the trajectory of changes with age, most significantly, the onset

of changes between female and male brains. In female brains, 44.2% of genes showed significant changes between the ages of 6 months and 9 months, and the changes were overall indicative of decreased bioenergetic function and increased amyloid dyshomeostasis. Protein analysis revealed that among six major estrogen and progesterone receptors, only ER β exhibited a significant change at this early transition in female brains. In stark contrast, in male brains, only 5.4% of genes were significantly changed in the same period. Subsequent changes in female brains were relatively small; however, in male brains, most changes occurred between the ages of 12 months and 15 months and the majority of changes were upregulation, suggesting a possible adaptive response to the aging process. Taken together, these data indicate that female and male brains follow profoundly disparate paths as they age, and female brains appear to undergo a much earlier aging process than male brains, in which ER β may play a role.

Among the genes involved in energy metabolism, significant changes associated with the earliest transition in female brains spanned across all functional domains examined, including insulin/IGF signaling, glycolysis, PDH and TCA cycle, ETC and OXPHOS, redox, lipid and ketone metabolism, mitochondrial biogenesis, mitochondrial dynamics, transport, and apoptosis, indicating a system-level bioenergetic decline. Bioinformatics analysis revealed that IGF1 appeared to serve as a central node in the overall decreased bioenergetic network associated with the transition from the age of 6 months to 9 months in female brains, which is consistent with the well-characterized properties of IGF1 as a master regulator of metabolism in both central and peripheral systems (McRory and Sherwood, 1997; Wit and Walenkamp, 2013). In the brain, IGF1 plays a significant role in diverse neural activities from early development to adult neurogenesis, to neuronal survival, and to cognition (Benarroch, 2012; Torres-Aleman, 2010). In particular, IGF1 has been well recognized for its role in the regulation of cellular bioenergetics and energy production. IGF1 signaling regulates multiple targets in glucose metabolism, including facilitation of the translocation of glucose transporters from the cytosol to the plasma membrane, thereby facilitating cellular glucose uptake and subsequent ATP generation (Bassil et al.,

2014; Yin et al., 2013). Moreover, IGF1 polymorphisms have been associated with increased risk for AD (Vargas et al., 2011; Wang et al., 2012), further highlighting the importance of IGF1 signaling in the maintenance of neurological health. Thus, it can be expected that sustaining IGF1 signaling and the downstream metabolic cascades through physical or pharmacological interventions holds promise to maintain the brain under a bioenergetically robust state, and as a result, offers a therapeutic benefit against the onset of metabolic deficits that could lead to the development of AD.

In addition to IGF1 signaling, another major change of particular significance was clusterin (CLU), which exhibited a 1.84-fold decrease in expression at the 6 to 9-month transition in female brains.

Bioinformatics analysis revealed that CLU may play a role in the regulation of both bioenergetic and amyloid metabolism in the brain. CLU, also known as apolipoprotein J, is a highly conserved glycoprotein that has been demonstrated as a pro-survival regulator in a variety of pathological events, including cancer progression and neurodegeneration (Bertram and Tanzi, 2010; Nuutinen et al., 2009; Rizzi and Bettuzzi, 2010; Sala et al., 2009). Recently, two independent genome-wide association studies and many follow-up studies have demonstrated a strong association between CLU genetic variants and increased risk for late-onset AD (Harold et al., 2009; Lambert et al., 2009). Moreover, recent studies have shown that CLU may play a role in the regulation of glucose metabolism (Daimon et al., 2011; Kim et al., 2011), and serve as a cellular biosensor of oxidative stress (Trogakos, 2013). CLU has also been shown to regulate amyloid metabolism by limiting amyloid peptide misfolding and facilitating their transport across the blood-brain barrier (Charnay et al., 2012; Yu and Tan, 2012). These earlier studies, along with our current findings, warrant further in-depth investigations of the roles of CLU in both normal neurological health and in the etiology of AD as well as its potential as a therapeutic target for AD prevention and early intervention.

The major question that remains to be determined is what could have caused the earliest transition in female brains. Could it have resulted from a reproductive status change, in particular, a reproductive transition similar to what occurs in humans, i.e., the transition from premenopause to perimenopause? In general, reproductive cycle irregularity in lab rodents, including both rats and mice, begins after 6 months (the age of “retired breeders”) and is significantly increased after 8 months, although some animals may be still actively cycling until 15 months of age (Gosden et al., 1983; Nelson et al., 1981). Regrettably, the animals used in this study were not monitored for their cycling profiles, so a conclusive correlation could not be drawn between the changes in the brain and the reproductive phenotype. Nevertheless, based on our recent analysis in which we found that approximately 50% of lab rodents at 9-10 months of age are irregular cyclers, it is reasonable to postulate that the 9-10-month female mice group used in our analysis were likely to be a heterogeneous population composed of both regular and irregular cyclers and they could have been at different estrous stages within the cycle. Despite the possibility that these mice differed in their reproductive profiles when tissues were collected, their brains exhibited greatly consistent expression patterns as shown in Fig. 2 and Fig. 3, suggesting that the changes observed between the ages of 6 months and 9 months in female mice brains cannot be simply related to the reproductive status or levels of classical sex hormones, such as 17β -estradiol and progesterone. With respect to the possible role of sex hormone receptors, it remains to be determined how significantly a decrease in ER β could contribute to the changes at this earliest transition in female brains. Together, these uncertainties warrant further in-depth investigations of potential contributors beyond the traditional concepts of reproductive aging and classical sex hormones that could play a major role in modulating sex-specific brain aging processes.

In summary, the purpose of this study was to determine how female and male brains age differently, specifically in two major areas that are most significantly affected in preclinical development of AD. The study presents several strengths and novelties. Technically, the state-of-the-art qRT-PCR-based TLDA

gene profiling technology provided us with not only a throughput but also a robust approach to investigate our hypotheses-driven questions at a system level. Scientifically, different from several prior studies that explored a similar subject, including the study conducted by Xu et al in which the researchers focused on late aging in mice (6, 16, and 24 months) (Xu et al., 2007), our study targeted an early aging window in mice (6, 9, 12, and 15 months). Our findings provide strong evidence indicating that female and male brains follow profoundly dissimilar trajectories as they age. The earliest transition indicative of the onset of a hypometabolic phenotype in female brains could serve as an important mechanistic rationale for female susceptibility to AD; and timely intervention at this transition could potentially halt the progression of metabolic deficits and ultimately reduce the risk of developing AD in women.

Disclosure Statement

All authors declare that there is no conflict of interests.

ACCEPTED MANUSCRIPT

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Table 1: Estrogen and progesterone receptor protein expression changes from 6 months to 9 months in female brains^a

Receptor	Fold Change (9mo vs 6mo)	P-Value
ER α	0.71 \pm 0.14	0.073
ERβ	0.70 \pm 0.10	0.047*
GPR30	0.77 \pm 0.18	0.164
PRA	0.95 \pm 0.11	0.397
PRB	0.85 \pm 0.13	0.182
PGRMC1	1.08 \pm 0.17	0.345

^a Western blots and graphs are included in Fig. S1

Figure Legends:

Figure 1. Female and male mice hippocampal gene expression changes with age. In female brains, 44.2% (73 genes, 23 increase, 50 decreases), 10.9% (18 genes, 3 increase, 15 decrease), and 6.1% (10 genes, 9 increased, 1 decreased) of genes showed significant changes between the ages of 6 months and 9 months, 9 months and 12 months, and 12 months and 15 months, respectively. In contrast, in male brains, 5.4% (9 genes, 2 increase, 7 decrease), 5.4% (9 genes, 4 increase, 5 decrease), and 27.3% (45 genes, 35 increase, 10 decrease) of genes were significantly changed from the ages of 6 months to 9 months, 9 months to 12 months, and 12 months to 15 months, respectively.

Figure 2A. Female and male mice hippocampal gene expression aging profile comparison. The heat maps display the relative expression levels of 85 genes with Ct < 35 analyzed by TLDA #1 (list of genes are included in Table S1); 4 mice per age group; red indicates high expression, green indicates low expression.

Figure 2B. Female and male mice hippocampal gene expression aging profile comparison. The heat maps display the relative expression levels of 80 genes with Ct < 35 analyzed by TLDA #2 (list of genes are included in Table S2); 4 mice per age group; red indicates high expression, green indicates low expression.

Figure 3. Hippocampal gene expression changes between the ages of 6 months and 9 months in female mice. The heat maps display the relative expression levels of genes that were significantly changed from 6 months to 9 months in female mice, including 36 genes from TLAD #1 (upper panel) and 37 genes from TLDA #2 (lower panel); 4 mice per age group; red indicates high expression, green indicates low expression. The volcano plots display fold changes (X-axis) with P-values (Y-axis) from 6 months to 9

months in female mice on all genes analyzed by TLDA #1 (upper panel) and TLDA #2 (lower panel); each dot represents a gene; red indicates upregulated genes, green indicates downregulated genes; dots that fall above the horizontal blue line indicate significantly changed genes ($P < 0.05$), dots that fall below the blue line indicate non-significantly changed genes ($P > 0.05$).

Figure 4. The IPA-derived molecular network with the most relevance to gene expression changes between the ages of 6 months and 9 months in female mice. Molecules (or nodes) in the network are displayed as various shapes that indicate the functional class. Focus molecules (colored molecules) refer to genes from the dataset. Red indicates significantly upregulated genes ($P < 0.05$), green indicates significantly downregulated genes ($P < 0.05$), gray indicates non-significantly changed genes from the dataset ($P > 0.05$), and white indicates molecules added from the Ingenuity Knowledge Base; color intensity indicates the degree of up or downregulation. Lines (or edges) connecting molecules indicate biological relationships; solid lines indicate direct interaction, dashed lines indicate indirect interactions, and circular lines indicate self-referential relationships that arise from the ability of a molecule to act upon itself; the types of arrows indicate specific biological relationships and the directionality of the interaction.

Figure 5. Hippocampal insulin-like growth factor 1 (IGF1) and clusterin (CLU) exhibited significant changes at both gene and protein levels at the transition from 6 months to 9 months in female mice. 4-5 mice per age group; * $P < 0.05$ and ** $P < 0.01$.

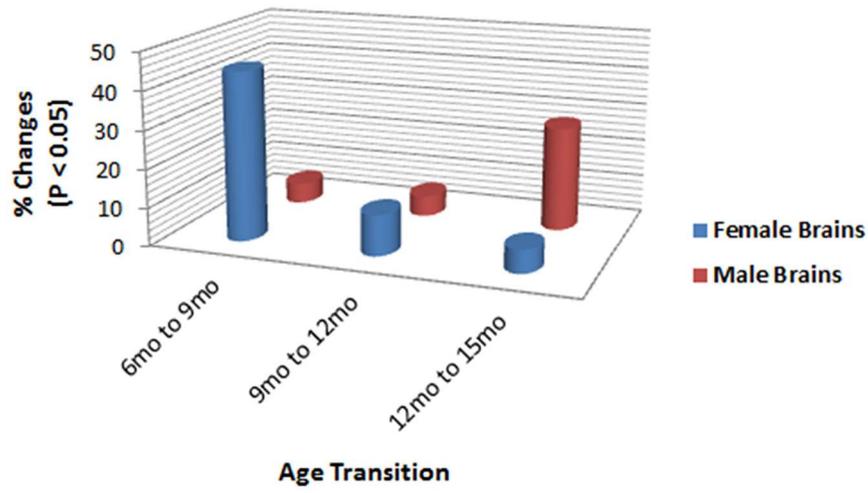
Supporting Information

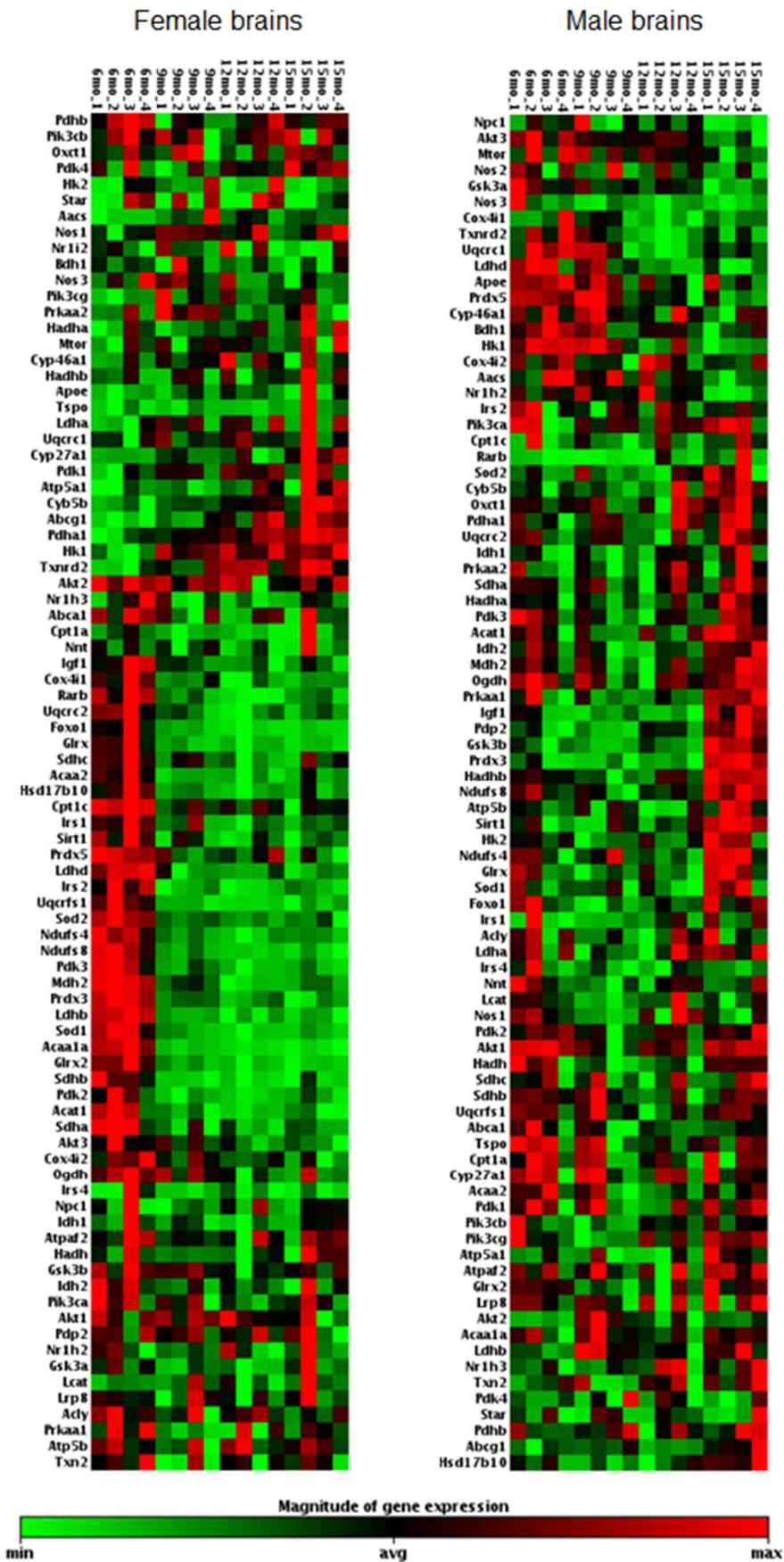
Table S1: TLDA #1 – Taqman gene expression assays

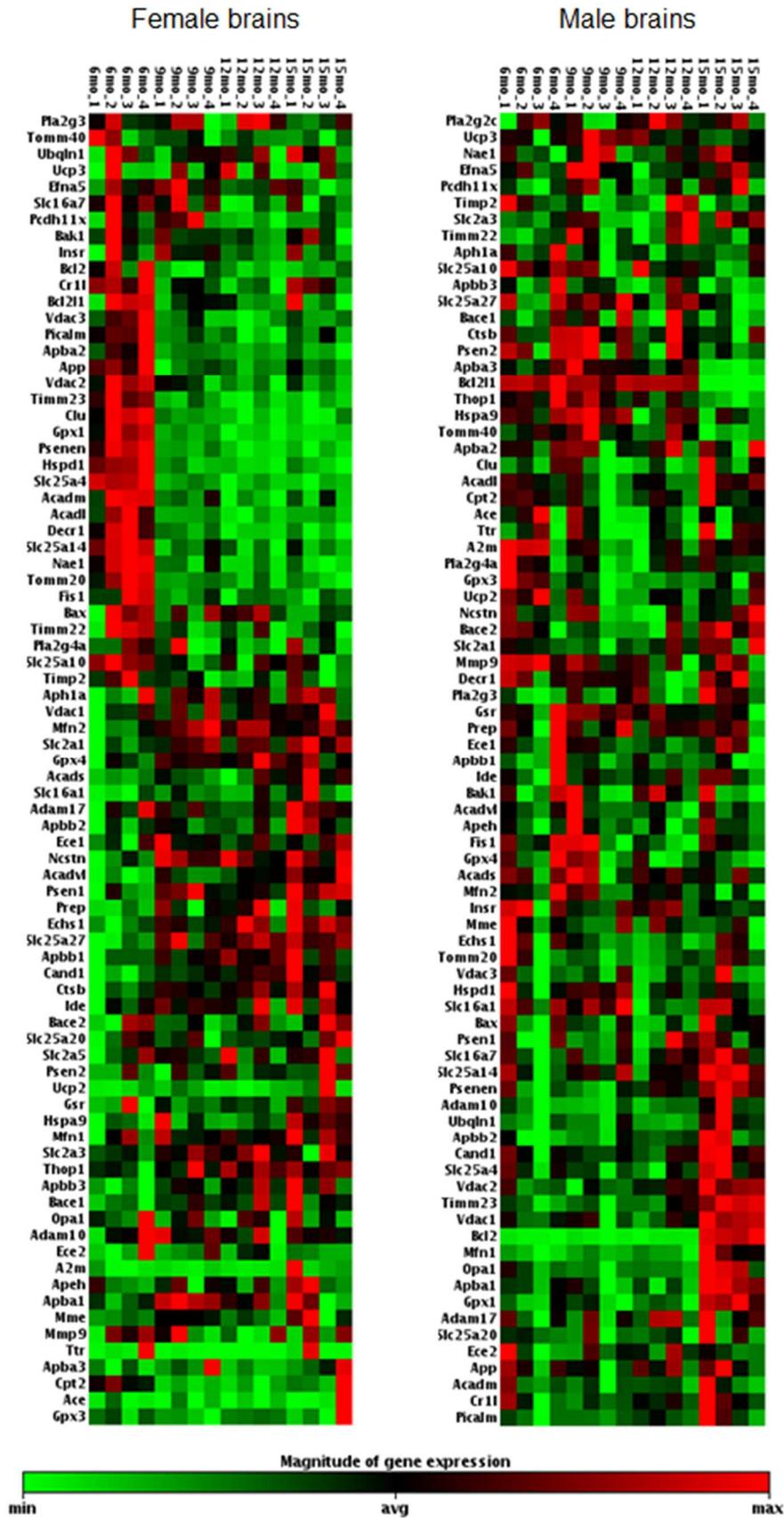
Table S2: TLDA #2 – Taqman gene expression assays

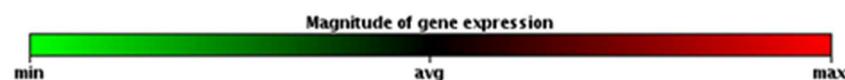
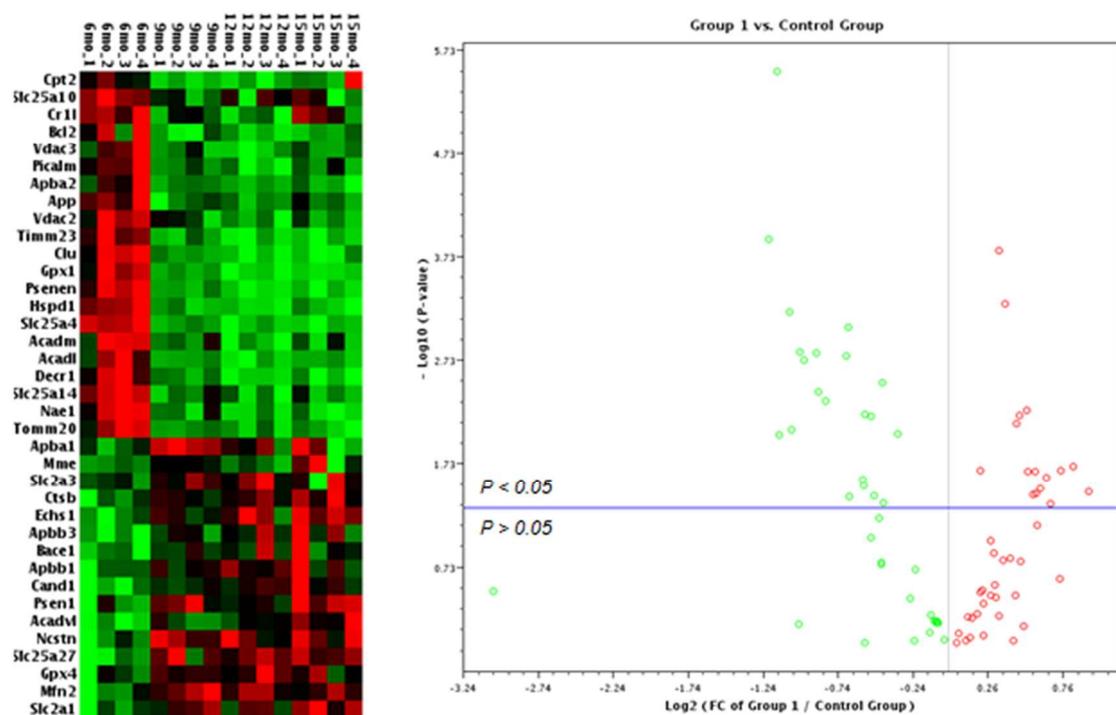
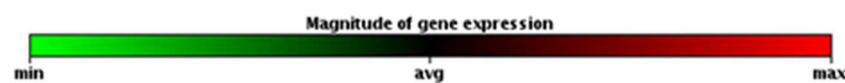
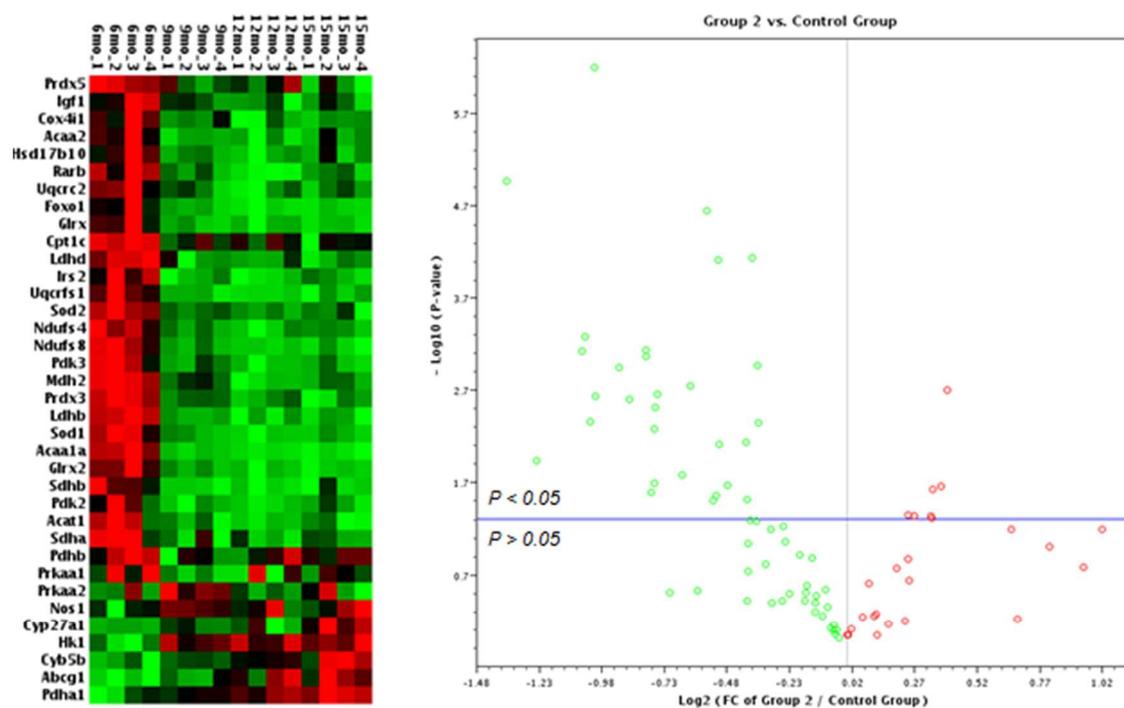
Table S3: Gene expression fold changes and P-values

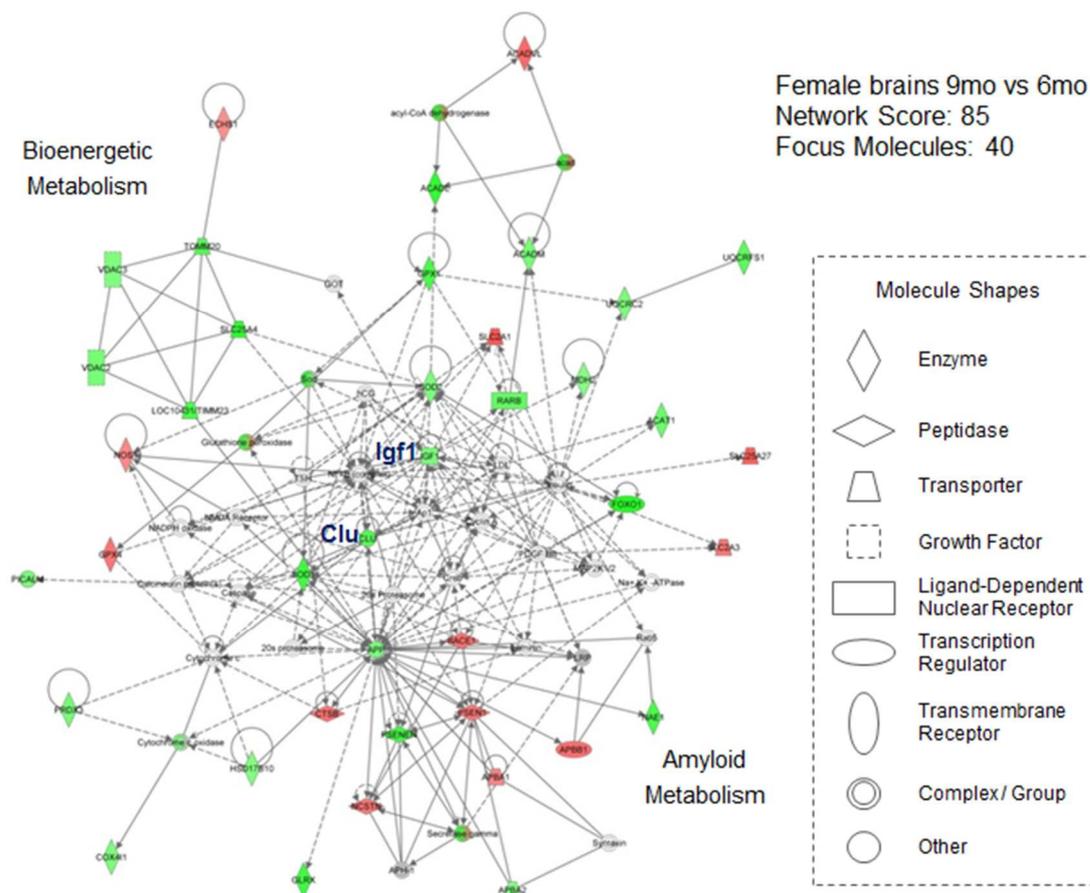
Figure S1: Estrogen and progesterone receptor protein expression changes from 6 months to 9 months in female brains

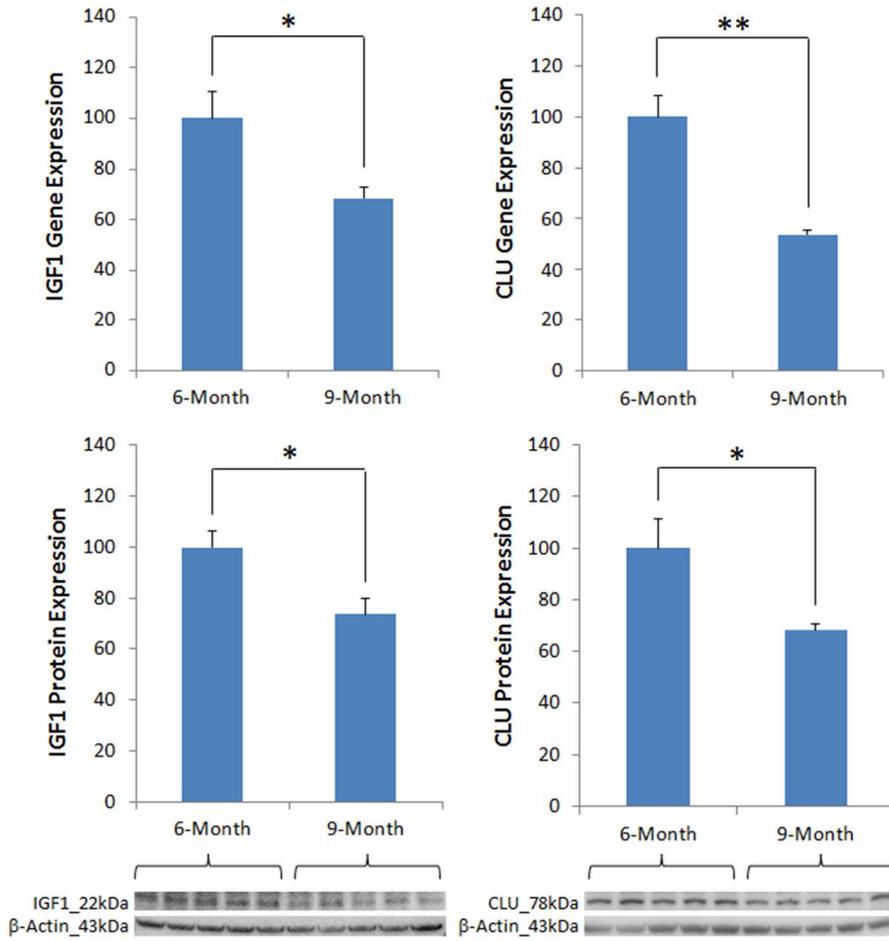






Female brains 9mo vs 6mo ($P < 0.05$)





Highlights:

1. Female and male brains follow profoundly dissimilar trajectories as they age.
2. Female brains undergo age-related changes much earlier than male brains.
3. Early changes in female brains signal the onset of a hypometabolic phenotype at risk for AD.
4. Timely intervention at the onset of early changes could potentially halt the progression of metabolic deficits and ultimately reduce the risk of developing AD in women.