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Identifying A β -specific pathogenic mechanisms using a nematode model of Alzheimer's disease

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Abstract

Multiple gene expression alterations have been linked to Alzheimer's disease (AD), implicating multiple metabolic pathways in its pathogenesis. However, a clear distinction between AD-specific gene expression changes and those resulting from non-specific responses to toxic aggregating proteins has not been made. We investigated alterations in gene expression induced by human β -amyloid peptide (A β) in a *Caenorhabditis elegans* Alzheimer's disease model. A β -induced gene expression alterations were compared to those caused by a synthetic aggregating protein to identify A β -specific effects. Both A β -specific and non-specific alterations were observed. Among A β -specific genes were those involved in aging, proteasome function, and mitochondrial function. An intriguing observation was the significant overlap between gene expression changes induced by A β and those induced by Cry5B, a bacterial pore-forming toxin. This led us to hypothesize that A β exerts its toxic effect, at least in part, by causing damage to biological membranes. We provide *in vivo* evidence consistent with this hypothesis. This study distinguishes between A β -specific and non-specific mechanisms and provides potential targets for therapeutics discovery.

1. Introduction

More than 35 million people are estimated to live with dementia worldwide, with prevalence expected to double every 20 years (Alzheimer's Disease International, 2009, Prince et al.,

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The authors of this manuscript claim no actual or potential conflicts of interest. To the best of our knowledge, neither the University of Colorado at Boulder nor the University of Wisconsin – Milwaukee have any contracts or any other sources of direct financial gain relating to this work.

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2013). Among all dementing disorders, Alzheimer's disease (AD) is the most prevalent, with estimated 24 million cases globally and 5.5 million in the United States (Ferri et al., 2005). The immense social and economic burden of AD and other dementing disorders necessitates prompt action at the level of governments, public health authorities, and research institutions. AD is characterized clinically by deterioration of cognitive functions, memory loss, behavioral changes, and motor dysfunction (Cavallucci et al., 2012, Crews and Masliah, 2010). Characteristic cellular changes in AD include loss of dendritic spines, synaptic dysfunction and loss, neuritic dystrophy, and ultimately neuronal cell death (Cavallucci et al., 2012, Crews and Masliah, 2010). It has been established that the deposition of beta-amyloid ($A\beta$) oligomers in the senile plaques formed in the brains of Alzheimer's patients is a hallmark of the disease, and so is the formation of intracellular neurofibrillary tangles rich in a hyperphosphorylated form of the microtubule associated protein *tau*.

Data from numerous studies supported a central role for $A\beta$ in the pathogenesis of AD (Cavallucci et al., 2012, Crews and Masliah, 2010). Yet, the exact triggers that initiate the pathological pathways culminating in neuronal cell death remain elusive. A better understanding of the initial molecular events that initiate AD-associated pathologies is crucial for the design of therapeutic interventions. Furthermore, the distinction between AD-specific molecular pathology and general misfolded protein response may be of equal importance. A logical approach to exploring AD pathogenesis at the molecular level is to identify gene expression changes associated with the disease. In this respect, microarrays provide a useful tool to simultaneously evaluate differential expression of thousands of genes in diseased cells and tissues.

There have been several studies aimed at profiling gene expression in AD using human brain tissues or mouse models. From these studies, links have been established between AD and abnormalities in multiple metabolic processes. These include, but may not be limited to, transcription, translation, proliferation, apoptosis, response to oxidative stress, mitochondrial function, chronic inflammation, protein processing and clearance, calcium homeostasis, synaptic function, cytoskeleton organization, and protein phosphorylation (Colangelo et al., 2002, Loring et al., 2001, Ricciarelli et al., 2004, Selwood et al., 2009). There have been several reports of AD-associated gene expression changes identified in postmortem brain tissues (Emilsson et al., 2006, Loring et al., 2001, Ricciarelli et al., 2004). Although these studies documented gene expression alterations pertinent to multiple metabolic pathways, the chronological order of the molecular pathological events involved could not be revealed. Consequently, these studies have not described the initial molecular triggers of the disease and often a distinction between a cause and an effect was not clear. The use of animal models enabled time course studies, in which gene expression changes were studied longitudinally as the pathology progressed. A study done using a PDAPP mouse model investigated gene expression changes in the early and late stages of $A\beta$ deposition. The same study also investigated gene expression changes in the absence of APOE (Selwood et al., 2009). However, a comparison between gene expression changes in the PDAPP mouse and a comparable model expressing a different aggregation-prone protein has not been done, making the $A\beta$ -specificity of the observed changes unclear.

The goal of this study is to elucidate A β -specific gene expression changes and discover putative pathogenic pathways triggered by A β toxicity. Using our inducible A β -expression worm model, we could acutely induce A β expression and study gene expression changes across a time course. A worm strain expressing soluble GFP (sGFP), which showed no gross pathological phenotypes, was used as a normal control. To discern A β -specific changes from those that could be caused by any aggregating protein, we also tested gene expression changes in worms expressing the non-disease associated, aggregation-prone protein GFP::degron (GFP_{deg}), which when expressed in worms causes a similar pathological phenotype to that caused by A β . Here, we show differential gene expression related to biological processes previously linked to AD. These include aging, unfolded protein response, mitochondria function, proteasome function, signal transduction, immune responses, cytoskeleton organization, cell cycle, growth rate regulation, and transport. Our data suggest that all of these processes were induced by A β -specific processes, except mainly the unfolded protein response and immune responses. We also identify a significant overlap between the response of *C. elegans* to transgenic A β expression and exposure to the Cry5B pore-forming toxin, implicating membrane damage as a component of A β toxicity *in vivo*.

2. Methods

2.1. Construction and propagation of *C. elegans* strains

We used three worm strains; CL4176, CL2337, and CL2179, which carry the human A β , GFP_{deg}, and sGFP transgenes, respectively. GFP_{deg} is a synthetic protein whose expression in worms causes pathologies that phenocopies those caused by A β expression (Link et al., 2006). sGFP was used as a negative control. All three transgenes were inducibly expressed in body wall muscles using a temperature-inducible, *smg-1^{ts}*-dependent system that we have previously described (Link et al., 2003, Link et al., 2006). Transgenes were engineered to bear long 3' untranslated sequences that target their transcripts to *smg-1*-dependent degradation. Since these animals carry a temperature sensitive mutant of *smg-1*, transgene mRNA transcripts are spared at non-permissive temperatures allowing for transgene expression. The choice of body wall muscle was made for two reasons: 1) A β and GFP_{deg} expression in body wall muscles produced a severe paralysis phenotype that could easily be scored to evaluate toxicity, 2) the lack of an easy-to-score worm strain with neuronal A β expression. Worms were grown using previously described protocols (Hassan et al., 2009). Briefly, synchronous eggs were prepared using hypochlorite solution and were incubated at 16°C (permissive temperature) for 36 hours. At permissive temperatures, SMG-1^{ts} is active and transgenes are silenced due to rapid degradation of their mRNAs. Animals were then shifted to a 25°C incubator to deactivate SMG-1^{ts} and allow transgene expression. Animals were harvested right before temperature upshift and 4, 8, 12, 16, and 20 hours later. Harvested animals were washed once with S-basal and pelleted animals were snap-frozen in liquid nitrogen. It is worth noting that the *smg-1^{ts}*-dependent silencing of transgenes used in this study is somewhat leaky, allowing for low levels of transgene expression at permissive temperatures (Data not shown).

2.2. Microarrays

RNA purification and microarrays were done as previously described (Hassan et al., 2009). Briefly, RNA was purified using RNeasy Kits (Qiagen) and the integrity of the purified RNA was evaluated by visualizing the 16S and 28S bands on an agarose gel, and using a bioanalyzer (data not shown). We had a single array per condition for time points T0, T4, and T20, and 3–5 arrays per condition for time points T8, T12, and T16 (Table 1). Biotinylated cRNA was synthesized using an Affymetrix kit (Affymetrix, Inc., Santa Clara, CA) using manufacturer's standard protocols. Briefly, 5 µg of total RNA from each sample were used to prepare cDNA by reverse transcription. Next, single stranded cDNA was converted into double stranded cDNA by second strand synthesis. The resulting double stranded cDNA was used as a template for *in vitro* transcription in the presence of biotinylated UTP and CTP to produce biotinylated cRNA. Hybridization and scanning were done at the microarray core facility of University of Colorado at Boulder. Raw intensity data extracted from CEL files was blotted in a box and whisker format using Expression Console version 1.1 (Affymetrix, Inc., Santa Clara, CA). Individual raw probe intensities (i.e. probe cell intensity) and the distribution of their ratios relative to the median probe across all chips were used to identify chips with median intensities that are dramatically different from the rest of the arrays (Fig. S1). Array 2179-8-3 (one of the four repeats of the 8th hour time point of sGFP worms) was clearly more divergent from median than all other arrays. The divergence of this array compared to all others was further demonstrated by Expression Console-generated signal histograms depicting either raw linear data summarized by MAS5 or quantile-normalized, log2 data summarized by either RMA or PLIER algorithms (Fig. S1). Principal component analysis was performed on MAS5-summarized, non-normalized data using GeneSpring GX version 11.0.2 (Agilent Technologies, Santa Clara, CA). Blotted data of the three principal components that accounted for most of the variability (45, 27, or 17%, respectively) among all arrays showed clear divergence of array 2179-T8-3 from all others (Fig. S1). Based on these findings, array 2179-T8-3 was excluded from further analysis.

2.3. Choosing an algorithm to summarize probe set intensities

We performed the analysis using five algorithms; MAS5, Robust Multichip Analysis (RMA), RMA with GC correction (GCRMA), Probe logarithmic Intensity Error Estimation (PLIER), and the algorithm developed by Cheng Li and Wing Hung Wong (Li and Wong, 2001). All chips were normalized using the Quantile algorithm. We used two criteria to judge the performance of each algorithm; control genes' coefficient of variation (CV) and the significance (measured by *p*-values) of gene expression changes known to occur in our worms. Control genes included housekeeping genes, hybridization controls, and labeling controls (Table S1), while genes with known expression changes in our worms included *aip-1* (Hassan et al., 2009), *tbb-6* (data not shown), *hsp-16.2* (Fonte et al., 2002, Link et al., 1999), and *hsp-70* (Magrane et al., 2004). First, we ranked summarization algorithms by the percentage of control genes that met a CV cutoff across all arrays included in the study. We found that PLIER yielded the highest percentage of probe sets that passed the CV cut-off overall and within the labeling control probe sets, and ranked second for both house-keeping genes and hybridization controls. RMA ranked first on housekeeping genes and second on

labeling controls and overall. MAS5 was the only algorithm that when used, 100% of the probe sets of hybridization control genes passed the cut-off. GCRMA performed poorly (Table 2). Second, we examined the *p*-values associated with each of the four genes known to change in our worms and we found that *tbb-6* and *hsp-70* were significantly altered with *p*-values at or below 0.02 and 0.04, respectively, regardless of the algorithm used. Similarly, *hsp-16.2* barely missed a *p*-value cutoff of 0.05 by all algorithms, except using RMA that showed a *p*-value of 0.05. However, *aip-1* showed heavy dependence on the summarization algorithm used, with a corrected *p*-value lower than 0.05 obtained only using LiWong algorithm, and the highest *p*-value obtained using GCRMA (Table 3). We concluded that the summarization algorithm used has a clear impact on the identification of differentially expressed genes. Based on our data, we decided to exclude GCRMA mainly due to the high variance in control gene expression levels across different arrays, and the particularly high *p*-value assigned to *aip-1*, a known differentially expressed gene. The remaining four algorithms were used to generate lists of differentially-expressed genes, and only genes that were common to all four lists were considered for further analyses.

2.4. Confirmation of selected gene expression changes using quantitative reverse-transcription, real-time polymerase chain reaction

Quantitative reverse-transcription, real-time polymerase chain reaction (qPCR) based on relative quantitation was employed to confirm a selected number of gene expression findings from our microarray analysis. RNA was purified using RNeasy kits (Qiagen) from worm cohorts independent from those used for microarray studies. We included 3 biological repeats for each of the time points tested. Primers were designed using Primer3 program (Koressaar and Remm, 2007, Untergasser et al., 2012) and qPCR was done using an ABI Prism 7000 or a StepOne PlusTM PCR system (Life Technologies Corporation, Grand Island, NY). SYBR Green master mixes were purchased from Life Technologies. Primer sequences are shown in table S2. Housekeeping genes were selected using NormFinder Software (Andersen et al., 2004). Five genes (*ama-1*, *gpd-3*, *rps-7*, *act-2*, and *eft-2*) were tested and the best combination of two genes was found to be that of *ama-1* and *rps-7* (Fig. S2). Target gene expression levels were normalized by dividing expression values by the corresponding geometric means of housekeeping genes' expression values. To confirm our microarray data, we tested gene expression changes of 20 genes using qPCR. Eleven genes had at least one probe set differentially expressed by all four summarization algorithms (MAS5, RMA, PLIER, and LiWong), 5 had at least one probe set differentially expressed by some but not all algorithms, and 4 were not differentially expressed. We compared microarray and qPCR data of CL2179 and CL4176 across the whole time course (i.e. T0–T20) for 13 genes, and at T8 and T16 for 7 genes. Among the genes we compared across the entire time course were a group of heat shock protein genes (*hsp-16.1/11*, *hsp-16.2*, *hsp-16.41*, and *hsp-16.48/49*). Congruence with microarray data was demonstrated in the up-regulation of these genes (two-way ANOVA *p*-value < 0.0001) (Fig. S2). Overall, congruence between qPCR and microarray studies was demonstrated for 14 out of 20 genes (Table S3, Fig. S2–S6). Two additional genes (*tmi-1* and *tmi-4*) were differentially expressed by qPCR (*p*-value < 0.0001), while in the microarray studies they were differentially expressed by some, but not all algorithms. Accordingly, they were considered unchanged by our criteria (1.5-fold change by all 4 algorithms with an ANOVA *p*-value < 0.05) for differential expression by

microarrays. However, the direction of the change remains consistent using either qPCR or microarrays. Another gene, *ttr-23*, was up-regulated using either detection technology, but qPCR failed to show statistical significance. For *pqn-18* and *pqn-32*, qPCR showed slight, but statistically significant change (ANOVA p-value < 0.0001), which was not captured by the microarray studies.

2.5. Calculating significance of gene enrichment

The significance of gene enrichment was calculated using either GeneSpring or Excel. In either case, hypergeometric distribution was calculated to indicate the probability that an observed overlap between a gene list and a subpopulation of the total number of genes tested was due to random chance alone. The sizes of the gene list, the total population, and the subpopulation in interest were all considered in calculating the probability. In calculating hypergeometric distribution, the total number of genes tested was considered the population size (m), the subpopulation of interest (e.g. genes that belong to a specific gene ontology term) was considered the total number of possible successes (k), and the overlap (j) between a gene list of interest (n) and the subpopulation (k) was considered the actual number of successes. Therefore, hypergeometric distribution is the probability that, by random chance alone, a gene list of size “n” may overlap by “j” genes with a subpopulation of the size “k”, given a total gene population of “m”.

2.6. Construction of A β -expressing *Escherichia coli* (*E. coli*) and subsequent exposure of *C. elegans*

The A β ₁₋₄₂ sequence was cloned between the *Nco*I and *Sac*I sites of the pBAD gIII vector (Invitrogen), resulting in plasmid pCL241 (sequence verified). This plasmid was transformed into *E. coli* strain LMG, allowing tightly controlled arabinose induction of A β (verified by immunoblot). Mid-logarithmic phase LMG/pCL241 was induced by arabinose for 2 hours, then reporter worm strain KWN117 (*vha-6::mCherry*) was fed cells from the induced culture for 4 hours before imaging.

2.7. RNA interference and paralysis assays

RNAi and paralysis scoring were performed as previously described (Hassan et al., 2009). Briefly, worms were fed HT115 *E. coli* carrying either the RNAi construct or a control vector from hatching to adulthood. Synchronous egg populations were collected from these worms and were incubated at 16°C (permissive temperature at which A β or GFP_{deg} expression is minimal) for 36 hours in the presence of the same bacteria. Worms were then shifted to a 25°C incubator to induce transgene expression. Scoring paralysis started 14 to 22 hours after transgene induction, dependent on the strain.

3. Results

3.1. A β -induced differential gene expression and the overlap with GFP_{deg}-induced gene expression changes

Differential gene expression was determined by comparing expression levels at a given time point between experimental strains (A β and GFP_{deg}) and the control strain (sGFP). A β -induced changes were compared to those induced by GFP_{deg} to determine A β -specific

effects. We found that transgenic A β altered the expression levels of 1315 transcripts, of which more than 950 transcripts were A β -specific and 364 were also altered by GFP_{deg} (ANOVA *p*-value 0.05). The probe set differential expression data is summarized in figure 1, with the corresponding numbers of transcripts shown in table 4.

3.2. Functional analysis of differentially expressed genes

To predict metabolic pathways affected by A β and identify A β -specific pathways, we analyzed lists of differentially expressed genes in two ways. First, we looked for metabolic pathways that were significantly enriched in the A β and GFP_{deg} gene lists. Pathway enrichment was performed using GeneSpring Single Experiment Analysis (SEA) to search the 1407 and 1360 probe sets induced by A β and GFP_{deg}, respectively (*p*-value = 0.03). Sixteen pathways were found significantly enriched in the A β list, while only two pathways were enriched in the GFP_{deg} list. Notably, aging, DAF-2/insulin signaling, mitochondrial unfolded protein response, and proteasome degradation pathways were among the significantly enriched pathways in the A β gene list, but not in the GFP_{deg} list (Fig. 2). Next, we addressed Gene ontology (GO) enrichment in the A β and GFP_{deg} gene lists. This analysis generally replicated the pathway enrichment analysis, with significantly enriched GO terms in the A β -altered gene lists (*p*-value < 0.001) related to aging and lifespan determination, stress response, cell cycle, regulation of growth rate, immune response, locomotion, cytoskeleton organization, and development and reproduction (Table S4). Similar GO term enrichment was observed among A β -specific genes (Table S5), with some exceptions. First, stress response genes were not enriched among A β -specific genes, presumably because those genes are general misfolded protein response genes that were induced by both A β and GFP_{deg}. Second, endocytosis and nucleic acid metabolism genes were enriched among A β -specific, but not the whole pool of A β -induced genes. We also observed enrichment of genes related to development and reproduction both among all A β -induced genes and A β -specific genes. This could have resulted from the delayed development of A β -expressing animals compared to their control counter parts. Interestingly, there were only a few GO term enrichments among the GFP_{deg} genes. Those were mainly of genes related to stress response and immune response (Tables S6 & S7).

3.3. A β -specific effects on expression of chaperone proteins

Up-regulation of several heat-shock proteins (HSPs) during the time course study was observed in both A β and GFP_{deg} animals, as expected based on our previous studies (Link et al., 2003, Link et al., 2006). These include the hsp16/hsp20/alphaB-crystallin family genes *hsp-16.1/hsp-16.11* and *hsp-16.48/hsp-16.49*, and the chaperone protein-encoding gene *hsp-70*. Interestingly, temporal differences are evident between the A β and GFP_{deg} animals (Fig. 3), as accumulation of HSP transcripts continues to increase over the time course of the experiment in A β -expressing animals, but plateaus in animals expressing GFP_{deg}. We have previously demonstrated that expression of A β in *C. elegans* results in the accumulation of ultrastructurally abnormal mitochondria (Fonte et al., 2011), as has been observed in other models and AD brains (Ferrer, 2009, Mancuso et al., 2009). Unexpectedly, expression of *hsp-6* (a mitochondrial Hsp70-class chaperone) and *hsp-60* (a mitochondrial GroE/Hsp-60-class) chaperone were strongly depressed at early time points relative to controls expressing

soluble GFP (Fig. 3). These mitochondrial chaperone transcripts did not show significant changes in the GFP_{deg}-expressing animals.

3.4. A β -induced differentially expressed genes include genes related to membrane damage

Pathway and gene ontology analyses are intrinsically limited by previously defined categorization, opening the possibility that coherent gene expression responses may be missed. Previous A β structure/function studies undertaken in the inducible transgenic *C. elegans* model have supported the hypothesis that A β toxicity results, at least in part, from the ability of this peptide to form membrane-damaging oligomers (Fonte et al., 2011). However, intrinsic membrane damage and repair are not recognized GO terms (<http://www.geneontology.org>). Fortunately, the transcriptional response in *C. elegans* to a pore-forming toxin has previously been characterized in the Aroian laboratory, where microarray analysis was used to assay transcript changes in worms fed the *Bacillus thuringiensis* Cry5B toxin expressed in *E. coli* (Huffman et al., 2004b, Kao et al., 2011). We asked if differentially expressed genes in the A β animals were enriched for transcripts up-regulated by exposure to the Cry5B toxin. We found that Cry5B-induced genes were significantly enriched among the A β differentially expressed genes (hypergeometric distribution 1.15×10^{-52}). We have also seen significant enrichment of Cry5B genes in the A β -induced early and late gene populations (Table 5). Since these findings are consistent with a pore-forming pathogenic mechanism for A β , we sought to test this hypothesis directly.

3.5. Intestinal expression of A β mimics the pathological effects of a membrane-forming toxin

The degree of overlap in gene expression profiles between the inducible A β strain and worms fed the Cry5B toxin (Table S8) was particularly striking given that the pathology in worms fed *E. coli* expressing the Cry5B toxin appears to be restricted to the intestine, while transgenic A β expression is restricted to the body wall muscle. Additionally, overlapping genes include ones, such as *hmp-1* (α -catenin ortholog) and *jun-1* (c-Jun transcription factor ortholog) that had been linked to late-onset AD (Cummings et al., 2012, Los et al., 2011). We reasoned, if the similarity between A β -induced and Cry5B-induced gene modulations was truly due to the ability of A β to form membrane pores or in some way damage membranes, feeding worms A β might replicate some of the pathology observed for the Cry5B toxin. To test this directly, we engineered *E. coli* to express A β using the pBAD gIII expression vector, which produces secreted A β in response to arabinose induction. A robust pathological effect of exposing worms to Cry5B-expressing *E. coli* is endocytosis of the intestinal luminal membrane, which can be visualized using luminal membrane protein fused to a fluorescent reporter (Los et al., 2011). This induction of endocytosis is believed to directly reflect repair of pore-forming toxin-damaged membranes. We therefore exposed a transgenic worm strain (KWN117) expressing a *vha-6::mCherry* reporter to engineered *E. coli* expressing either A β or a control protein (calmodulin). As shown in Figure 4, a 4-hour exposure of fourth larval stage worms to control *E. coli* has no effect on the luminal distribution of the *vha-6::mCherry* reporter, while exposure to A β -producing *E. coli* strongly induces the formation of large endocytic vesicles.

3.6. A JNK-family mitogen-activated protein kinase protective against Cry5B and cadmium toxicity is also protective against A β , but not GFP_{deg} toxicity

KGB-1 is a JNK-family mitogen-activated protein kinase (MAPK) that has been shown to protect against pore-forming toxins and cadmium toxicity in *C. elegans* (Huffman et al., 2004a). Given the striking overlap between A β -induced differential gene expression and that caused by Cry5B, we thought to test the role of KGB-1 in A β toxicity. We show that *kgb-1* RNAi exacerbates the paralysis phenotype of A β animals, an effect that seems to be A β specific since *kgb-1* RNAi had no effect on the paralysis phenotype GFP_{deg} animals. This RNAi treatment had no effect on a non-transgenic control strain (Fig. 4), indicating that *kgb-1* knockdown does not cause paralysis on its own, yet it hypersensitizes the worms to A β toxicity.

4. Discussion

We have utilized a *C. elegans* AD model to study global gene expression changes associated with the transgenic expression of human A β in worms. Acute A β expression was triggered by temperature upshift and gene expression changes were assessed at time intervals before the onset of the severe paralysis phenotype characteristic of these transgenic worms. Since the misfolded protein response is invoked in multiple neurodegenerative disorders that are collectively characterized by protein aggregation and accumulation of misfolded proteins (Heller, 2010, Johnston et al., 1998), we thought to separate A β -induced gene expression changes from general misfolded protein response. For this purpose, we compared gene expression changes resulting from A β expression to changes induced by the expression of the non-disease associated, aggregation prone protein GFP_{deg}. Almost all aggregating proteins that have been studied *in vivo* (e.g., superoxide dismutase, α -synuclein, etc.) are associated with their own specific pathologies (Taylor et al., 2002), making them poor controls for aggregation *per se*. However, we have shown that a C-terminal addition of a 17 residue “degron” peptide to GFP renders this protein aggregation prone in both mammalian cells and *C. elegans* (Link et al., 2006). Thus, the GFP_{deg} protein represents an aggressively aggregating version of a normally innocuous protein, which is particularly well-suited for controlling for the general response to protein misfolding. We found that A β induced gene expression changes are consistent with accelerated aging, mitochondria abnormalities, and proteasomal dysfunction. Accelerated aging has been previously suggested by studies that showed an overlap between aging- and AD-associated gene expression changes in the human brain (Avramopoulos et al., 2011, Sætre et al., 2011).

A link between mitochondrial dysfunction and AD has been extensively documented in the literature (Casley et al., 2002, Celsi et al., 2009, Lunnon et al., 2012, Reddy et al., 2004, Selwood et al., 2009, Trushina et al., 2012, Zhu et al., 2012). In *C. elegans*, mitochondrial heat shock proteins (HSP-6 and HSP-60) are also linked to aging since their loss in RNAi experiments led to a progeria-like phenotype. These proteins are essential for mitochondrial biogenesis and energy production (Kimura et al., 2007). In this study, we demonstrate marked transcriptional down-regulation of both *hsp-6* and *hsp-60* in A β -expressing animals, a phenotype that could not be detected in GFP_{deg} animals. Abnormal mitochondrial morphology has been previously reported in A β -expressing worms (Fonte et al., 2011) and

in the brains of AD patients (Celsi et al., 2009). Interestingly, no morphological changes have been observed in the mitochondria of our GFP_{deg} animals (Link et al., 2006). Our findings suggest that A β affects mitochondrial integrity and function via an A β -specific mechanism that is not invoked by general protein aggregation or misfolding.

Additionally, our data implicated A β in membrane damage. A striking overlap between gene expression changes induced by A β expression in muscle and those induced by an intestinal exposure to the Cry5B toxin impelled us to test whether a JNK-family MAP kinase shown to protect against the bacterial toxin could also protect against A β . Our RNAi studies support the involvement of this gene (*kgb-1*) in protection against A β toxicity, which appears A β -specific since the paralysis phenotype of GFP_{deg} animals was not affected by *kgb-1* RNAi knockdown. Although a significant overlap between Cry5B and GFP_{deg} genes has been observed (data not shown), our *kgb-1* RNAi results suggest that Cry5B and A β are linked through a distinct mechanism. To the best of our knowledge, this study provides the first *in vivo* evidence supporting the hypothesis that pore formation or membrane damage may be a component of A β toxicity. These data support a link between A β -induced mitochondrial dysfunction and damage to biological membranes in the context of AD. Elevated reactive oxygen species (ROS) and disrupted calcium homeostasis, both induced by A β and linked to AD pathogenesis, lead to mitochondrial dysfunction. Mitochondrial dysfunction results in elevated ROS. ROS is associated with β -secretase up-regulation and lipid peroxidation, which may enhance A β production and intracellular accumulation, respectively (Galindo et al., 2010). The abundance of polyunsaturated fatty acids in neuronal cell membranes makes them particularly sensitive to lipid peroxidation (Ferrer, 2009). Membrane damage may contribute to the disruption of calcium homeostasis and mitochondrial dysfunction (Galindo et al., 2010). Lipid peroxidation, pore formation, and increased production and intracellular accumulation of A β may all combine to form a vicious positive feedback cycle.

Finally, we showed that A β effects on proteasome-associated transcripts were also A β -specific. The present study, as well as our previous work showing up-regulation of *aip-1*, a regulatory component of the 19S proteasome caps (Yun et al., 2008), in the A β animals, implicates proteasome dysfunction in A β toxicity. In the current study, *aip-1* differential expression was not captured due to the high stringency of the parameters used to select significant genes. However, we have previously confirmed *aip-1* up-regulation by quantitative real-time polymerase chain reaction (Hassan et al., 2009).

The *C. elegans* model used in this study was developed to assay components of A β toxicity, and clearly cannot capture many aspects of AD. Nevertheless, the extensive congruence between our findings and many known AD-linked gene expression changes (e.g. mitochondrial and proteasomal dysfunction) supports the relevance of this invertebrate model to AD. We identified pathological pathways specific to A β , and by extension AD, as well as others that are common between A β and general protein aggregation, highlighting potential targets that could be used for designing therapeutic interventions in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Some, but not all, A β -induced gene expression changes are specific to A β .
- A β -specific genes include aging, mitochondrial, and proteasomal function genes.
- A β may cause damage to biological membranes as part of its pathogenic mechanisms.

Fig. 1.

Longitudinal analysis of A β -specific gene expression changes. Differentially expressed genes in the A β strain were determined using four probe set summarization algorithms (MAS5, RMA, PLIER, and LiWong). Shown here are the numbers of differentially expressed probe sets obtained using each algorithm and the overlap between these lists. Probe sets that were differentially expressed regardless of the algorithm used are highlighted in yellow (center of each venn diagram). Shown here are (A) gene expression changes over the entire time course, including probe sets that were differentially expressed at one or more

time points in either direction, probe sets that were (B) up-regulated or (C) down-regulated eight hours post-induction, (D) up-regulated or (E) down-regulated sixteen hours post-induction, and those that were (F) up-regulated or (G) down-regulated eight, twelve, and sixteen hours post-induction. Note: Some genes were represented by multiple probe sets in the chips used in this study.

Fig. 2.

Metabolic pathway enrichment in A β - and GFP_{deg}-induced genes. Pathway enrichment was done using GeneSpring Single Experiment Analysis (SEA) to search 1407 A β -induced and 1360 GFP_{deg}-induced probe sets. These probe sets correspond to 1315 A β -altered and 1237 GFP_{deg}-altered gene transcripts. The percentages of pathway transcripts found in a list of differentially expressed genes (blue) and other pathway transcripts (red) are plotted for significantly enriched pathways. The numbers of transcripts are indicated in the corresponding bars.

Fig. 3. Temporal transcript accumulation of specific chaperones in Aβ and GFP_{deg} animals
 (A) Transcript levels for *hsp-16* and *hsp-70* cytoplasmic chaperones in Aβ animals (B) Transcript levels for *hsp-16* and *hsp-70* cytoplasmic chaperones in GFP_{deg} animals. (C) Transcript levels for *hsp-6* and *hsp-60* mitochondria chaperones (*hsp-6* and *hsp-60* did not show significant changes in GFP_{deg} animals). There were two probe sets for *hsp-60* in the arrays used in this study: * probe set 176345_s_at, ** probe set 175619_s_at.

Fig. 4. A β -induced phenotypes parallel those observed after exposure to the Cry5B pore-forming toxin

(A & B) Intestinal exposure to A β results in the formation of endocytic vesicles (arrows) in a *vha-6::mCherry* reporter *Caenorhabditis elegans* strain. RNAi knockdown of *kgb-1* augments A β toxicity (C), but does not show any effect on GFP_{deg} toxicity. CL802 worms are control worms that do not express either of the toxic proteins. *kgb-1* RNAi in these worms does not cause any detectable pathology, indicating that *kgb-1* RNAi is not on its own toxic. Depicted in (B) is the mean number of endocytic vesicles counted in 6 representative animals. Paralysis data represent three independent assays, and the number of

worms used in each assay (n1, n2, n3) is shown. Error bars represent the standard deviation. Size bar: 50 micrometers.

Table 1

Samples and microarrays used in this study. RNA was purified from three worm strains; A β , GFP_{deg}, and sGFP (CL4176, CL2337, CL2179, respectively). All repeats were biological repeats, with each chip hybridized to a unique sample. Only one sample/chip was used for T0, T4, and T20 of each strain. Multiple (3–5) samples/chips were used for time points T8 through T16.

Time Points	Number of Repeats		
	CL2179 (sGFP)	CL4176 (A β)	CL2337 (GFP _{deg})
Time of temperature upshift (T0)	1	1	1
Four hours after upshift (T4)	1	1	1
Eight hours after upshift (T8)	3	4	4
Twelve hours after upshift (T12)	5	4	5
Sixteen hours after upshift (T16)	4	4	4
Twenty hours after upshift (T20)	1	1	1

Table 2

Percentage of control genes passing a specified coefficient of variation cut-off by the summarization algorithm used. Numbers in parentheses represent the number of probe sets in each group of controls. The highest percentage in a row is shown with a black background, the second highest, the middle, and the second to lowest are shown with backgrounds of decreasing shades of grey, and the lowest percentage in a row is shown with a white background.

Probe Set	CV Cut-Off	MAS5	RMA	GCRMA	PLIER	LiWong
House-Keeping Genes (15)	0.05	67%	87%	60%	73%	73%
Hybridization Controls (9)	0.05	100%	67%	56%	89%	78%
Labeling Controls (12)	0.15	33%	58%	0%	67%	33%
Cumulative (36)		64%	72%	39%	75%	61%

Table 3
Corrected *p*-values of known differentially expressed genes obtained using five probe set summarization algorithms.

Gene Symbol	MAS5	RMA	GCRMA	PLIER	LiWong
<i>atp-1</i>	0.12	0.11	0.14	0.07	0.03
<i>tbb-6</i>	0.01	0.02	0.01	0.01	0.02
<i>hsp-16.2</i>	0.06	0.05	0.06	0.06	0.06
<i>hsp-70</i>	0.04	0.03	0.02	0.04	0.03

Table 4

Differential gene expression in A β and GFP_{deg} animals. Significant gene expression changes across the entire time course of A β induction was determined using ANOVA, with a corrected *p*-value cut-off of 0.05. Gene numbers are listed with the numbers of the corresponding probe sets in parentheses.

Time point	All A β	A β -specific	General misfolded protein response
Entire time course	1315 (1407)	957 (1009)	364 (398)
Up at T8	108 (117)	47 (50)	61 (67)
Down at T8	43 (46)	35 (38)	8 (8)
Up at T16	221 (233)	78 (81)	144 (152)
Down at T16	51 (54)	25 (27)	26 (27)
Up T8 - T16	30 (32)	18 (18)	12 (14)
Down T8 - T16	12 (12)	11 (11)	1 (1)

Table 5

Enrichment of membrane-damage responsive genes in the A β -induced genes.

Gene population	A β -specific probe sets	Probe sets overlapping with Cry5B	Percent overlapping	Hypergeometric distribution
Entire time course	1351	56	3.98	1.15×10^{-52}
Up at T8	94	23	19.66	9.08×10^{-36}
Up at T16	212	21	9.01	3.90×10^{-25}