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Neurobiology of Aging 32 (2011) 2319.e27–2319.e34

NEUROBIOLOGY OF AGING

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Gene expression reveals overlap between normal aging and Alzheimer's disease genes

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Received 15 September, 2009; received in revised form 23 March 2010; accepted 20 April 2010

Abstract

Alzheimer's disease (AD) is a common cause of dementia with a strong genetic component and risk sharply increasing with age. We performed two parallel microarray experiments to independently identify genes involved in normal aging and genes involved in AD using RNA extracted from the temporal lobe of 22 late onset AD and 23 control brain donors. We found that AD is accompanied by significant changes in the expression of many genes with upregulation of genes involved in inflammation and in transcription regulation and downregulation of genes involved in neuronal functions. The changes with healthy aging involved multiple genes but were not as strong. Replicating and strengthening previous reports, we find a highly significant overlap between genes changing expression with age and those changing in AD, and we observe that those changes are most often in the same direction. This result supports an overlap between the biological processes of normal aging and susceptibility to AD and suggests that age related genes expression changes might increase the risk of developing AD.

Keywords: Dementia; Gene expression; Aging; Microarray; Brain; Temporal lobe

Alzheimer's disease is the most common cause of dementia in the USA, affecting an estimated 5.2 million (Alzheimer's Association report 2008). With few exceptions of familial cases due to mutations in one of three known genes, *APP* (Goate et al., 1991), *PSEN1* (Sherrington et al., 1995) and *PSEN2* (Levy-Lahad et al., 1995), Alzheimer's disease (AD) has a late age of onset, most often after the age of 65. It presents with progressive loss of multiple cognitive abilities leading within an average of 8 years to severe dementia and death. Although it is a complex genetic disorder, late onset AD is in large extent due to genetic predisposition with a heritability calculated between 0.6 and 0.74 (Bergem et al., 1997; Gatz et al., 1997). Despite this major genetic influence only one gene, *APOE* encoding for apolipoprotein E, has been consistently shown to be involved in the risk for late onset AD (Strittmatter et al., 1993), while a few more genes with variants contributing significantly less to the risk are now emerging through recent large genome wide association studies (Harold et al., 2009; Lambert et al., 2009).

Like most cell types, neurons respond to normal or abnormal stimuli—such as those involved in a disease process—by setting in motion signaling cascades and modifying their internal and external microenvironment. Among the responses are changes in the priorities of the protein synthesis machinery, observed as changes in gene transcription and the levels of the relevant mRNAs. Measuring these changes can provide information on the nature of the genes that play a role in the disease process and allow comparisons with other physiological or pathological states. In some cases an observed difference in the levels of a particular mRNA between diseased and control tissue might reflect the primary defect that contributes to the risk. A challenge in this type of analysis is to distinguish between the primary and secondary alterations. Nevertheless, knowledge of gene expression changes involved in a disease may prove useful

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doi:10.1016/j.neurobiolaging.2010.04.019

in understanding the disease process and possibly exploring preventions and treatments. Further, gene expression profiling can itself be a means to test and develop new drugs (Gerhold et al., 2002). There are multiple array-based choices for surveying genome wide gene expression that differ in their content, the probe preparation methods, and the chemistry of the array surface. The most commonly used include laboratory developed cDNA arrays and commercial gene chip products from Affymetrix®, Illumina®, Amersham® Agilent® NimbleGen®, and other biotechnology companies. Such chips examine thousands of genes often covering much more than the well characterized genes in the genome and in some cases interrogating individual exons.

There have been many studies investigating gene expression changes in AD (Blalock et al., 2004; Colangelo et al., 2002; Dunckley et al., 2006; Emilsson et al., 2006; Ginsberg et al., 2000; Haroutunian et al., 2009; Kong et al., 2009; Liang et al., 2007; Liang et al., 2008; Loring et al., 2001; Parachikova et al., 2007; Ray et al., 2008; Ricciarelli et al., 2004). Among the multiple variables that can influence the results of such studies are the selection of tissue type or brain region, the expression analysis platform, and the analytical methods. Differences in these variables between different studies together with small sample sizes, stochastic and other variation, have often led to inconsistent observations. Most studies use one of two main approaches to the interpretation of their results. Some focus on the individual dysregulated genes and make hypotheses on the possible roles of the gene products in the disease process. Others identify groups of genes either by setting a significance threshold or through gene coexpression network analyses (Zhang and Horvath, 2005) and then examine the groups for excess representation of specific functional classes. Although such groups likely contain false positives, overall they are highly enriched for true positives and their composition can provide significant and reliable results. Although different platforms and analytical methods can lead to different results at the individual gene level, gene class enrichment is robust across platforms (Maouche et al., 2008) and while this approach does not identify specific target genes, it provides important insights into the possible disease mechanisms and consequences of the disease at the molecular level.

Our motivation for this study was two-fold. First, we wanted to provide new insights and add support to conclusions from previous gene expression studies of AD. Second, we wanted to test for an overlap between gene expression changes in AD and in normal aging as suggested by a previous report (Miller et al., 2008), a phenomenon that we think could have great importance to our understanding of the genetics of AD. In two parallel studies we examined the gene expression profile of Broadman area 22 (superior temporal lobe), an area strongly affected by AD pathology, in 22 ADs cases and 23 controls without brain pathology at death. The samples were split in two independent sets, one focusing in AD and using a subset of nine controls matched to the cases and another focusing on changes with age using the remaining 14 controls with a relatively wide spectrum of ages and no AD cases. We used the Illumina Sentrix HumanRef-8 Expression BeadChips that interogate 24,000 genes recognized by the National Center for Biotechnology Information (NCBI). We report on genes showing significant changes in AD, on functional enrichments among genes changing expression, and on a highly significant overlap between the two groups, an observation that we replicated using a third, public dataset.

1. Methods

2.1. Samples

We obtained 3-mm punch biopsies from the superior temporal lobe (Brodman area 22) of 22 deceased patients with confirmed AD pathology and 23 controls with no brain pathology. All cases and controls were of European descent and their ages, gender, and the time between death flash freezing of the brain slices (Post Mortem Delay; PMD) are shown on supplementary Table 1 together with Braak staging and CERAD scores for cases.

Samples were split in two sets, (i) the AD sample-set of 22 cases and nine controls with no significant differences in age, gender, PMD, or positioning on the Illumina Beadchips, and (ii) the AGE sample-set with a wider age range (35–93) consisting of samples free of pathology and with no significant correlations between age and PMD, gender, or placement on Illumina Beadchips. All the details pertaining to the sets are shown on supplementary Table 1. All samples were from brains collected by the Johns Hopkins Brain Resource Center (courtesy of the director Dr Juan Troncoso).

2.2. Transcript measurements

To measure transcript abundance we used the Illumina Sentrix HumanRef-8 Expression BeadChips (Illumina, San Diego, CA 92,121-1,975, cat. no. 11201828) containing 24,000 genes recognized by NCBI at the time of production. We extracted total RNA using Trizol (Invitrogen, Carlsbad, CA 92,008, cat. no. 15596-026) with additional purification on RNA easy columns (Qiagen, Valencia, CA 913,555, cat. no. 74104). We assessed the quality of total RNA on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and 0.5 μg of total RNA from each sample was labeled by using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX 78,744–1,832, cat. no. IL1791) in a process of cDNA synthesis and *in vitro* transcription. We generated and labeled single-stranded RNA (cRNA) by incorporating biotin-16-UTP (Roche Diagnosics, GmbH, Mannheim, Germany, cat. no. 11388908910) and hybridized (16 hours) a total of 0.85 μg of biotin-labeled cRNA to the BeadChips. The hybridized biotinylated cRNA was de-

tected with streptavidin-Cy3 and quantitated using Illumina's BeadStation 500GX Genetic Analysis Systems scanner. The primary Illumina data were returned from the scanner in the form of an '.idat' file which contains single intensity data values/gene following the computation of a trimmed mean average for each probe type represented on the array by a variable number of bead probes. We performed preliminary analyses of the scanned data using Illumina BeadStudio software which returns a detection call D based on a comparison between the intensity of a single probe and the intensities of a large number of negative control beads built-in to the BeadChip arrays (D = % above negative/ 100, 1 = perfect, i.e. the intensity value of a gene is greater than all the intensities for every negative control tested). Any gene consistently below $D = 0.98$ was eliminated from further analysis, leaving data for 11,326 named genes expressed in temporal lobe for analysis. Normalization of the expression values to account for differences in input RNA, processing, labeling, etc. was performed by Z-transformation on each sample/array on a stand-alone basis (Cheadle et al., 2003).

Replications using SYBR-green real time detection with Applied Biosystems reagents (Foster City, CA, cat. no. 4,312,704) and an ABI 7,900 sequence detection system (Applied Biosystems) were performed on newly extracted RNA from the same tissue sample. After the examination of melting curves excluding nonspecific PCR products, relative quantification of each sample was performed using a standard curve from standardized dilutions of a reference RNA. Measurements were normalized against the average of two housekeeping genes (*M-RIP* and *POLR2*) selected from a study that identified AD-appropriate reference genes using a neuroblastoma cell line that models aspects of Alzheimer's disease in culture (Hoerndli et al., 2004). Ratios to the reference were log transformed resulting in normally distributed values.

2.3. Data analysis

We used Z-normalized expression values of each transcript as the dependent variable in a generalized linear model that included disease status, age, gender, and PMD for the AD sample-set and age, gender, and PMD for the AGE sample-set. The analyses were performed in R (version 2.4.1, cran.r-project.org) using the "glm" function for generalized linear models. Expression change was investigated in the AGE sample-set while changes with disease were investigated separately in the independent AD sample-set. We also used a set of public data, those from a study by Myers et al. (Myers et al., 2007), as a third independent set for the effects of age. We analyzed the temporal lobe data of that dataset using the rank invariant normalized data supplied by the authors as an outcome and including age, PMD, gender, and sample source in the generalized linear model.

The results were then parsed on excel spreadsheets, where quantile quantile (Q-Q) plots were generated, false discovery rate (FDR) was calculated from the *p*-values following the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995), results of the AGE sample-set and AD sample-set were matched by gene and compared in parallel, and gene lists were generated for functional enrichment analyses.

We used the expression data analysis tools provided by the Panther classification system web site (www.pantherdb.org/tools/genexAnalysis.jsp) (Mi et al., 2007; Thomas et al., 2003) for enrichment analyses for specific gene functions. We used lists of genes showing changes in the generalized linear models described above at the chosen FRD thresholds against the reference set of all genes whose transcript was positively detected by the array (thus all genes that could possibly be included in the list of genes with expression changes). The Panther web site performs a modified Bonferroni correction which accounts for the nesting of child gene ontology terms below parent terms. The *p*-values shown on Table 1 are Bonferroni corrected through this method. The significance of overlaps between lists of genes with expression changes was assessed using standard 2×2 tables of counts of genes present or absent in each list and were tested for independence by chi-square tests.

3. Results

All 45 cases and controls provided good quality RNA without significant degradation, as shown by gel electrophoresis and analysis on the Agilent 2100 Bioanalyzer, and were successfully processed and analyzed by the Illumina BeadStudio software. Expression of 11,326 named genes was positively detected by BeadStudio and their data were processed as described in the materials and methods. Figure 1 shows Q–Q plots of the distribution of *p*-values for expression changes with AD and with age and supplementary Figure 1 shows volcano plots for the two datasets. There is clear inflation of low *p*-values in the AD sample-set and 1,031 genes were found dysregulated at an FDR < 0.05. In the AGE sample-set there is also an inflation of signals at *p*-values between 0.05 and 0.001 which, however, does not continue at lower *p*-values, suggesting multiple true signals yet small effects that cannot reach strong statistical significance. In agreement with this, the FDR is 0.4 at $p < 0.0415$ but does not improve much thereafter (lowest FDR is 0.345 at $p < 0.0086$). Therefore we chose this relatively relaxed FDR (0.4) to define genes regulated with aging. Although this is expected to include a significant number of false positives it also has the highest enrichment for true positives we can achieve for such a large group, consisting of 1,174 genes more than half of which are expected to be true positives. Of these genes, 604 (51.4%) showed decreased expression with age. Gender and PMD did not show strong effects with the exception of four genes, all located on the Y chromosome, that showed a strong gender effect on both sample-sets, reaching an FDR of less than 0.05 in the larger

Table 1
Enrichments in genes expressed higher in AD

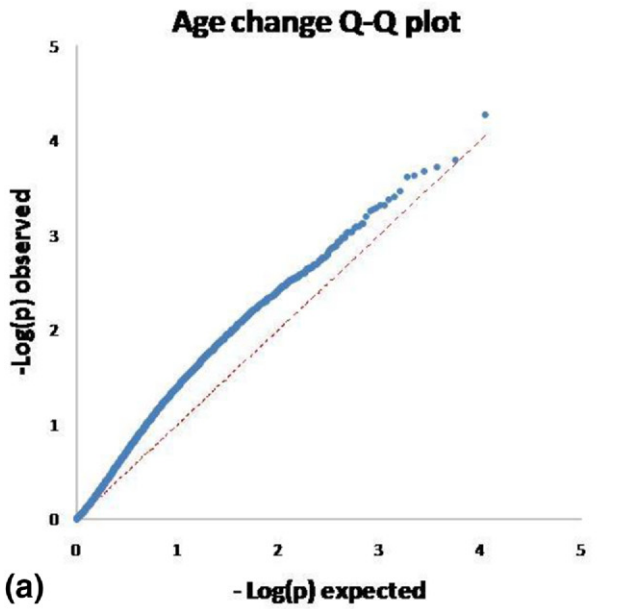
Higher expression in AD	Category	Number of genes	Fold enrichment	Corrected significance
FDR < 0.05–504 genes				
Interleukin signalling pathway	Pathway	36	2.3	**
mRNA transcription	Biol Proc	222	1.4	***
Oncogenesis	Biol Proc	68	1.87	***
Nucleoside, nucleotide and nucleic acid metabolism	Biol Proc	353	1.2	**
Hematopoiesis	Biol Proc	18	3.3	**
mRNA transcription regulation	Biol Proc	167	1.4	**
Cell structure and motility	Biol Proc	132	1.4	**
Immunity and defense	Biol Proc	137	1.4	**
Macrophage-mediated immunity	Biol Proc	21	2.5	*
Cell proliferation and differentiation	Biol Proc	119	1.3	*
Developmental processes	Biol. Proc.	211	1.2	*
Transcription factor	Mol. Funct.	221	1.3	***
Nucleic acid binding	Mol. Funct.	282	1.3	***
Lower expression in AD	**Category**	**Number of genes**	**Fold enrichment**	**Corrected significance**
FDR < 0.05–526 genes				
Neuronal activities	Biol. Proc.	117	1.9	***
Synaptic transmission	Biol. Proc.	63	1.9	***
Nerve-Nerve synaptic transmission	Biol. Proc.	23	2.7	**
Ion channel	Mol. Funct.	57	1.7	**
Voltage-gated ion channel	Mol. Funct.	31	2.1	*
Neuropeptide	Mol. Funct.	11	3.6	+

Functional enrichment for genes expressed at higher or lower levels in Alzheimer's disease (AD) cases. The category column refers to the ontology categories used for the analysis.
$^{+}$ $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All p values are Bonferroni corrected.

AD sample-set. One of them also reached FDR < 0.05 for gender regulation in the smaller AGE sample set analyses. Among the genes that were downregulated with age there was significant enrichment for those whose products are involved in pre-mRNA processing ($p = 0.0025$), splicing ($p = 0.004$), and for ribosomal protein genes ($p = 0.00001$). No significant enrichment for functional classes was observed among genes upregulated with age.

The analysis of the AD sample set revealed many highly significant differences with 1,031 genes showing change in expression at FDR < 0.05, 51% of these showing reduced expression in AD cases (see supplementary Table 2 for the complete list). The upregulated genes were enriched for transcription factors ($p = 2.3 \times 10^{-3}$) and genes involved in nucleic acid metabolism($p = 0.023$). Downregulated genes were enriched for genes involved in neuronal activities ($p = 7.5 \times 10^{-3}$), specifically for voltage-gated ion channels ($p = 0.022$). Using a more relaxed FDR threshold of 0.2 which identified 1,804 downregulated and 1,602 upregulated genes, we were able to achieve much stronger statistical evidence of enrichment shown on Table 1.

We then examined the hypothesis that the set of genes changing with age might be enriched for genes involved in AD. Using the same FDR levels above (0.2 for AD and 0.4 for age) 3,406 of the 11,326 genes change expression levels in AD and 1,174 change with age. The two groups shared 451 genes, significantly more than expected to overlap by chance ($p = 4.5 \times 10^{-11}$). The significance remained strong if we used the stringent FDR of 0.05 for AD with 166 overlapping genes ($p = 2.1 \times 10^{-10}$). Strikingly, in all but one of the 166 (and in 95% of the 451) overlapping genes the change with increasing age was in the same direction with the change in AD. This is illustrated on the scatter plot in Figure 2. To exclude any systematic error in our two parallel experiments we further examined a public dataset that was informative for gene regulation with age, that of Myers et al. (Myers et al., 2007). We downloaded the data and analyzed them as described in our materials and methods for the effect of age. There were in total 9,743 transcripts with present calls in at least two-thirds of individuals. This dataset provided somewhat more significant results than ours, presumably because of the larger sample size (131 samples from the temporal lobe) with 10 transcripts reaching an FDR < 0.1, yet none an FDR < 0.05. The Myers et al. study was performed on an Affymetrix array and among the transcripts called present we could match 6,368 to those with present calls in our sample-sets. Of the 86 transcripts overlapping at FDR < 0.4 between our AGE sample set and the Myers et al. set 76 (88%) showed change in the same direction for both sample sets, supporting the validity of the results. The Myers et All dataset included 690 genes at FDR < 0.4, 415 decreasing with age and enriched in peptide hormone genes ($p = 0.033$) and 275 increasing with age with no significant enrichments. We compared the



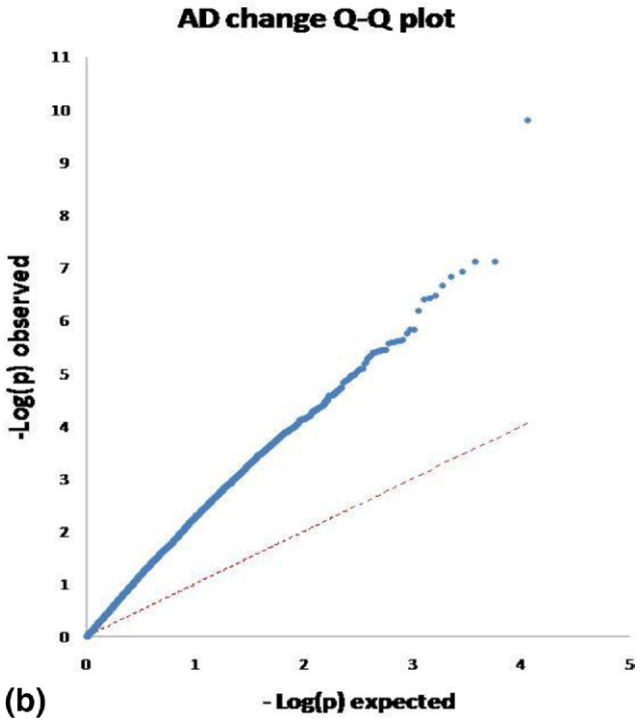


Fig. 1. (A) Quantile Quantile plot for the observed *p*-value distribution for the effect of age on gene expression compared with the expected null distribution (B) As in Figure 1(A) for the effect of AD on gene expression.

genes changing in AD from our AD sample set (FDR $<$ 0.2) to the genes changing with age from the Myers dataset (FDR $<$ 0.4) and again we found a significantly high overlap between the two sets. Of the 6,368 genes in common 1304 (our AD sample set) changed in AD, 503 with age (Myers dataset) and 150 were in common ($p = 6 \times 10^{-8}$). Once again the vast majority (133, 89%) were changing in the same direction with age and ad.

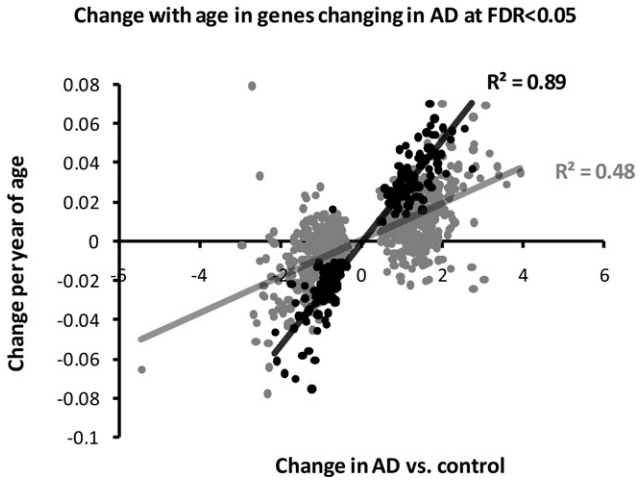


Fig. 2. Scatter plot of effects on gene expression (linear model parameter estimates) of genes that change significantly in AD at FDR $<$ 0.05. Black and gray dots correspond to genes that change significantly with age at FDR $<$ 0.4 or not. A strong directional correlation is observed, stronger for genes with a significant age effect.

We compared our results with the list of genes reported by Miller et al. to be overlapping in AD and aging with at least one significant probe set (Miller et al., 2008) (found on their suppl. Table 6). In their results, strong excess of genes with same the directionality of change was also evident, yet not as striking as we observe. The overlap of their list of 558 genes changing both with age and AD with our list of 451 (FDR $<$ 0.2 and 0.4 for AD and age respectively) was 31 genes. For 23 of those (74%) the results of both studies were in the same direction both for aging and AD. Only one of the remaining genes showed completely discordant results between studies (see suppl. Table 3). Finally we compared our list of age regulated genes with those reported by Lu et al. (Lu et al., 2004) in their Supplementary Table 2. All 19 genes that we could match by RefSeq name from their table and were present in our list of age regulated genes at FDR $<$ 0.4 showed the same direction of effect, further validating our results.

We experimentally validated our microarray results by extracting new RNA from the AD sample-set and measuring expression by real time PCR. We tested eight genes, three with FDR $<$ 0.05 (*VGF, RBP4, ADCYAP1*) and five with FDR 0.2 (*SOX10, NPTXR, VMD2, SP1, BDNF*). In all eight cases the new measurement showed an effect in the same direction as the microarray. In five cases including all three at FDR $<$ 0.05 the result replicated with at least nominal significance (see suppl. Table 4). The remaining three showing the same direction but without nominal significance are likely due to the platform differences, noise introduced by harvesting new tissue and extracting new RNA and likely also include false positives.

4. Discussion

We have performed a large microarray based gene expression study exploring the effects of age on gene expression and comparing it to gene expression changes in AD. We found that the effects of aging on gene expression is relatively subtle yet it involves multiple genes. Our separate study of transcript abundance differences between AD affected brains and unaffected controls showed multiple significant differences with high statistical confidence. Most importantly, when comparing the results of the expression study on AD with that on aging we found that more genes than expected were affected by both and almost always in the same direction, i.e. genes whose expression goes down with age are often found to be lower in AD affected brains and vice versa. This result had highly significant statistical support and was observed almost as strong when we analyzed data on the effect of aging from a completely independent publicly available dataset using a different platform. In fact the overlap was stronger when age effects were calculated using the Myers et al. data ($\sim$ 50% higher than expected by chance) than when using our own ($\sim$ 30% higher), reflecting perhaps the higher number of control brains sampled.

We provide a list of 1,030 genes in our supplementary material (suppl. Table 2) that shows differences in expression in AD at FDR $<$ 0.05. This is a high confidence list likely to include mostly true positives and it includes multiple genes that have been previously reported. Some of these genes could reflect primary changes, i.e. they could be responsible for the development of the disease, but most are likely secondary changes, in response to the disease process. Our functional enrichment results are consistent with previous literature and provide additional support for specific functions while expanding the observations to Brodmann area 22. We found an enrichment for genes in the interleukin signaling pathway, immunity and defense, and specifically macrophage mediated immunity among the genes with higher expression in AD (Table 1) which supports links between inflammation and AD (DeLegge and Smoke, 2008; McGeer and McGeer, 1998; Wyss-Coray, 2006). We also found an enrichment in transcription factors and other genes involved in transcription, likely reflecting the induction of cellular responses by the disease process. Among the genes with lower expression in the AD brain, we found enrichments in genes involved in synaptic transmission, ion channels, and generally genes involved in neuronal activities. Our results replicate, strengthen, and expand previously published similar findings (Katsel et al., 2005; Papassotiropoulos et al., 2006), elaborating on important aspects of the AD process.

The result that we found most striking and also carried the strongest statistical support was the overlap between genes dysregulated with AD and genes that change expression with age. Such an overlap was first described by Miller et al. (Miller et al., 2008), who performed a systems level analysis of transcriptional changes in Alzheimer's disease and normal aging. Here we provide strong replication using multiple independent datasets and we show a strong directionality of this phenomenon. This significantly increased overlap could result from many different underlying links between aging and AD related genes, and is of particular interest because advanced age is the most significant AD risk factor. It is possible that changes in some genes' expression with age, although part of the normal aging process, can also lead to increased vulnerability to AD. It is possible that for a subset of such genes changes might happen faster for some individuals—perhaps due in part to genetic variation—leading to increased vulnerability. As these individuals would end up in our case group they could produce the observed results. The overlap might also reflect a globally accelerated aging process in the people who are vulnerable to AD, which could be due to genes, environment, or both. It must be noted that the small effects on gene expression observed with age forced us to adapt an FDR of 0.4, meaning that a significant number of false positives are included in the results. This might reduce the confidence in the validity of individual gene results however it does not reduce the importance of the highly significant overlap which would be expected even stronger if we could clear false positives off our lists.

The genetic overlap of aging and AD has important implications for aging research. It would be useful to perform more and larger studies covering more brain regions and more patients and controls to confidently identify this set of overlapping genes. These genes are likely to be important to healthy aging and possibly primary culprits for vulnerability to AD, either of which would make them important targets for pharmacological intervention. The observed low effect of normal aging on gene expression which currently translates to low statistical confidence for individual genes underscores the importance of further research on expanded datasets, as defining the exact overlap between normal aging and AD could lead to significant breakthroughs in our understanding and our therapeutic approach to the disease.

Disclosure statement

The authors have no actual or potential conflicts of interest to disclose.

Acknowledgements

This work was supported by NIA grants to DA and SSB (RO1AG022099 and RO1AG021804) and an award from the Neurosciences Education and Research Foundation to D.A. We thank the Johns Hopkins Brain Resource Center and director Dr. Juan Troncoso for contributing the tissue samples studied here.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2010.04.019.

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