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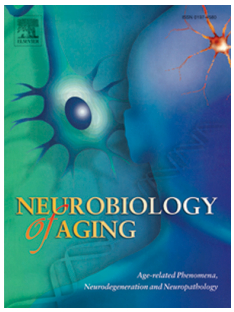

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Hippocampal transcriptome profiling combined with protein-protein interaction analysis elucidates Alzheimer's Disease pathways and genes

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ABSTRACT

Knowledge about the molecular mechanisms driving Alzheimer's disease (AD) is still limited. In order to learn more about AD biology, we performed whole transcriptome sequencing on the hippocampus of 20 AD cases and 10 age- and sex-matched cognitively healthy controls. We observed 2,716 differentially expressed genes, of which 48% replicated in a second dataset of 84 AD cases and 33 controls. We used an integrative network-based approach for combining transcriptomic and protein-protein interaction (PPI) data to find differentially expressed gene modules that may reflect key processes in AD biology. A total of 735 differentially expressed genes were clustered into 33 modules, of which 82% replicated in a second dataset, highlighting the robustness of this approach. These 27 modules were enriched for signal transduction, transport, response to stimulus and several organic and cellular metabolic pathways. Ten modules interacted with previously described AD disease genes. Our study indicates that analyzing RNA-expression data based on annotated gene modules is more robust than on individual genes. We provide a comprehensive overview of the biological processes involved in AD, and the detected differentially expressed gene modules may provide a molecular basis for future research into mechanisms underlying AD.

1. INTRODUCTION

Alzheimer's Disease (AD) is a neurodegenerative disorder hallmarked by progressive loss of memory, currently affecting over 40 million individuals worldwide (Prince, et al., 2013,Scheltens, et al., 2016). Previous studies have shown neurodegenerative changes in the hippocampus 15-20 years before symptom onset (Boyle, et al., 2013,Karran, et al., 2011,Murray, et al., 2011). The main pathological features are amyloid plaques and tau tangles throughout the brain (Braak and Braak, 1995,Holtzman, et al., 2016,Jellinger, 2008,Selkoe and Hardy, 2016,Thal, et al., 2014,Tomiyama, 2010). Multiple AD associated genetic loci have been identified, although their pathophysiological mechanisms remain largely unknown (Bekris, et al., 2010,Lambert, et al., 2013,Van Cauwenberghe, et al., 2016).

Transcriptomic studies on post-mortem AD brain tissue have been performed to further our understanding of AD biology (Kavanagh, et al., 2013,Sutherland, et al., 2011). Most of these studies report differentially expressed genes and pathways in brain tissue of AD cases compared to controls (Ashburner, et al., 2000,Gene Ontology, 2015,Ogata, et al., 1999). Most of these studies report a decrease in synaptic transmission, mitochondrial function and cytoskeleton biology. In contrast, an increase is often reported in immune response, inflammation and apoptosis in AD cases (Liang, et al., 2008,Ray and Zhang, 2010,Sekar, et al., 2015,Twine, et al., 2011). Recently, network-based analysis are utilized to provide more extensive and robust insights in these data, for example based on protein-protein interaction (PPI) data (Chi, et al., 2016,C. Humphries, et al., 2015,C.E. Humphries, et al., 2015,Kong, et al., 2015,Kong, et al., 2014). The largest amongst these studies investigated gene expression (measured with RNA arrays) in more than a thousand brain samples, spread across 19 regions in 125 individuals (Wang, et al., 2016). By performing gene co-expression analysis on AD cases of varying severity and non-demented controls they identified dysregulated gene modules and pathways. The study concluded that some of those originated from early disease stages and might reflect causal mechanisms, but also highlighted to use of gene modules rather than individual genes. In this study these modules are based on only co-expression type PPI data.

The goal of our study was to compare whole transcriptome sequencing of 20 AD cases with 10 age- and sex-matched cognitively healthy controls. We aim to identify differentially expressed genes and cluster these into functional gene modules using PPI data. We aim to replicate these differentially expressed genes, gene modules and functions in a second independent RNA sequencing dataset (van der Brug H, 2017) to determine the robustness of replication based on gene modules compared to individual genes.

2. MATERIAL AND METHODS

2.1. Data generation

Hippocampus samples were selected from the Netherlands Brain Bank for 20 AD cases (Braak and Braak, 1995,Mirra, et al., 1991) and matched for age and gender with brains from 10 non-demented cognitively healthy controls (Table 1). The dentate gyrus and cornu amonis were macro-dissected from the hippocampus tissue and total RNA was isolated using the manufacturer's protocol (Qiagen AllPrep RNA isolation, Cat No. 80224). Sequencing was performed after poly-A selection and TruSeq library prep at the Human Genomics facility (HUGE-F, www.glimdna.org) on a HiSeq2000 at 2x50bp. Data was processed per sample using trim-o-matic (v0.33), STAR (v2.3.0) (Bolger, et al., 2014,Dobin, et al., 2013), picard (v1.90) and fastQC (v0.11.3). Transcript quantification was performed using featurecounts (v1.4.3) against all 57,820 gene features in GENCODE (version date; 2013-12-05) (Harrow, et al., 2012,Liao, et al., 2014). For replication, dataset GSE95587 was downloaded from the Gene Expression Omnibus (GEO). This dataset contained raw RNA-seq counts of the fusiform gyrus for 84 AD cases and 33 controls and was processed in parallel to the discovery dataset in all subsequent steps (van der Brug H, 2017).

2.2. Data analysis

Counts were normalized using the edgeR (v3.8.6) trimmed mean of M-values (TMM) method to counts per million (CPM) values, and all low-abundant features were omitted (<1 CPM in 75% of samples). Principal components (PCs) were calculated using “prcomp” in R, and then plotted to visually identify sample outliers. Statistical analysis was performed per gene using the exacTest function in edgeR, correcting for age, gender and the first 2 principal components (McCarthy, et al., 2012,Robinson, et al., 2010). We combined FDR-corrected p-values and log fold changes to calculate a differential expression score; $\frac{-\log_{10} p_{(FDR)}}{10} * \frac{\sqrt{logFC * logFC}}{3}$. Genes with a DE score ≥ 0.10 are considered differentially expressed genes (DE-genes) and retained for further analysis. All steps were performed identically and separately for the discovery and replication datasets.

2.3. Protein-protein interaction (PPI) clustering

For all DE-genes, we extracted experimental, co-expression and database interactions scored ≥ 500 from STRING v10 (von Mering, et al., 2003). This network was imported to Cytoscape (v3.4.0) and subjected to the Markov Clustering Algorithm (MCL) in order to identify gene modules (Morris, et al., 2011,Smoot, et al., 2011). In short; MCL clusters graphical data to determine groups of genes (modules) with more interactions within the module than to the rest of the network (Enright, et al., 2002). This clustering method revolves around one main parameter which determines the module sizes; the inflation factor. We optimized the inflation factor to retain modules between 10-100 genes to allow for subsequent gene set enrichment analysis (Subramanian, et al., 2005). Each gene can only be assigned to a single module. Modules smaller than 10 genes are excluded. All steps are performed separately for the discovery and replication dataset.

2.4. Functional annotation of modules

For each identified gene module, enrichments for gene ontology biological processes (GOBP) were performed using Webgestalt (v27-1-17) (Gene Ontology, 2001,Ogata, et al., 1999). For GOBP enrichment the "noRedundant" terms were used. All enrichments were FDR (Benjamini-Hochberg) corrected, using a threshold of $p<0.05$ for statistical significance. Only the first three enriched GOBP-terms were extracted for each gene module. All three GOBP-terms for all gene modules were then pooled, and divided into shared common ancestor terms, denoted as GOBP-branches (Ashburner, et al., 2000,Carbon, et al., 2009). Therefore, each gene module can be annotated with three GOBP-terms, and their respective GOBP-branch. Modules from discovery and replication are divided in to the same GOBP-branches. They can thus be enriched for the same GOBP-term, or enriched for different GOBP-terms that are closely related by sharing a common ancestor term.

2.5. Replication of DE genes and modules

DE-genes and gene modules were generated separately for the discovery and replication datasets using the

exact same methodology. Replication of discovery modules is assessed by the number of overlapping genes and overlapping GOBP-terms within the replication modules. Different degrees of robustness of overlap between our data and the replication dataset were classified. Category 1) a gene module overlaps in genes and in GOBP-term(s) with a gene module from the replication dataset. Category 2) a gene module overlaps in GOBP-term(s), but not in genes with a replication module. Category 3) a gene module overlaps in genes with a replication module, but not in GOBP-term(s). When a module from discovery shares a parent GOBP-term with a replication module this was also considered replication.

2.6. Mapping known AD genes

We selected a list of 27 known AD risk genes, compiled from known AD GWAS loci and Mendelian causal genes (Lambert, et al., 2013,Van Cauwenberghe, et al., 2016). All experimental and database interactions between these 27 AD genes and the genes in discovery modules were extracted from STRING, using a cutoff of ≥500. An AD gene was considered to interact with a discovery module when it interacted with at least two of the genes in that module.

3. RESULTS

3.1. Study sample characteristics

The demographic data of the AD group did not differ from the control group, as shown in Table 1. As expected, mean brain weight, Braak and CERAD stages and post mortem delay differed significantly between AD cases and controls. On average 48,772,000 reads were sequenced per sample. All sequencing quality and alignment QC metrics were similar between groups. Two outliers were identified by principal components, driven by high expression of *TTR*. This gene is specifically expressed in the choroid plexus, which was confirmed using routine staining and both cases were excluded. The replication dataset GSE95587 consisted of fusiform gyrus from 84 AD cases and 33 controls and is described elsewhere (van der Brug H, 2017).

3.2. Differentially expressed genes and modules

A total of 2,716 genes was differentially expressed in the discovery dataset (DE score ≥ 0.1), as shown in Figure 1. Examination of known interactions between these DE-genes showed that 1,610 DE-genes shared one or more interaction(s). Using this interaction network, we clustered 735 discovery DE-genes into 33 discovery gene modules. The expression table and gene-module assignments can be found in supplemental Table 1. In the replication dataset, 2,490 DE genes were identified. A total of 1,311 DE-genes from the discovery dataset (48%) replicated in the replication dataset, as shown in Figure 2. From the interaction network of replication DE-genes, 653 DE-genes were clustered into 37 replication modules.

3.3. Functional annotation and replication of modules

Gene set enrichment analysis of each module resulted in three significantly enriched gene ontology biological processes per module in discovery and replication. These enriched GOBP-terms were pooled across all discovery and replication modules and assigned to eight main GOBP-branches; “Organic substance metabolic process”, “Signal Transduction”, “Transport”, “Regulation of biological process”, “Cellular metabolic process”, “Cellular component organization”, “Other metabolic processes” and “Response to stimulus”. The remaining

terms are grouped under a 9th branch; “Other biological processes”. Table 2 shows the three GOBP-terms for all discovery modules, their respective branches and category of overlap with the replication modules. Further details about these branches and overlap can be found in supplementary Figure 2.

Combined across all 33 differentially expressed gene modules in the discovery dataset, we identified 84 GOBP-terms (at maximum three per gene module, see Table 2). For 19 of the 33 discovery modules, the discovery module overlaps both in genes and GOBP-term with a replication module (overlap category 1), as shown in Figure 3. Another eight gene modules overlap a GOBP-term with a replication module, but do not overlap in genes (overlap category 2). Five modules overlapped in genes but did not overlap in GOBP-term with the same replication module (overlap category 3). A single module did not overlap in either genes or GOBP-term with the replication modules. This result brings the replication results of gene modules with the replication dataset at 73% when based on overlapping genes (category 1 and 3) compared with 82% based on overlapping GOBP-term(s) (category 1 and 2).

3.4. Interaction with AD genes

Of 27 known AD risk genes, 25 were expressed in the brain tissue that was studied. Three genes (11%) showed a DE-score of ≥ 0.1; *CD2AP* (score 0.18), *MEF2C* (-0.29) and *PTK2B* (-0.50), none of these were assigned to a module. Only *MEF2C* and *PTK2B* are replicated with a DE-score of -0.39 and -0.13, respectively. Ten AD genes interacted at least twice with a discovery module; *ABCA7*, *APP*, *BIN1*, *CELF1*, *CLU*, *HLA-DRB1*, *HLA-DRB5*, *MAPT*, *PICALM* and *PTK2B*, as shown in Table 2. Six AD genes interacted only once with a discovery module; *APOE*, *CD2AP*, *INPP5D*, *MEF2C, PSEN1* and *PSEN2*. Nine AD genes did not interact with any discovery module; *CASS4*, *CD33*, *CR1*, *FERMT2*, *MS4A6A*, *RIN3*, *SLC24A4*, *SORL1* and *ZCWPW1*.

4. DISCUSSION

Our study identified 2,716 differentially expressed genes (DE-genes) in hippocampus of 18 AD cases compared to 10 age- and sex-matched non-demented controls. Of these 2,716 DE-genes, 735 were clustered in 33 gene modules based on protein-protein interaction data. These 33 gene modules were assigned 84 gene ontology biological processes (GOBP-terms, at maximum three for each gene module) which together comprise nine main GOBP-branches. All nine branches were frequently observed in previous AD studies.

4.1. Replication by gene modules and GOBP-terms is more robust and identifies the most central AD changes

Replication of our results in an independent dataset (GSE95587, fusiform gyrus of 84 AD cases and 33 controls, (van der Brug H, 2017)) was based on different categories of overlap, reflecting the robustness of these overlapping processes in the underlying pathophysiology of AD. The finding that the majority of our gene modules falls into category 1 (n=19) indicates that the combined approach of GOBP annotated and PPI clustered gene modules identifies the most robust changes in AD gene expression. The gene modules in category 2 (n=8) and category 3 (n=5) might reflect some variability of gene expression between hippocampus in our study and fusiform gyrus of AD brains in the replication study.

The current comparative study supports the idea that the overlapping datasets based on gene modules or GOBP-term per module is more robust than based on overlapping genes only, as the overlap of all DE-genes (48%) can be improved by categorization into gene modules (72%), and even more by overlap based on GOBP-terms (82%).

4.2. GOBP branches represent common AD pathways

The nine main GOBP-branches are previously observed in literature of AD expression studies (Chi, et al., 2016,C.E. Humphries, et al., 2015,Liang, et al., 2008,Ray and Zhang, 2010,Sekar, et al., 2015,Twine, et al., 2011,Wang, et al., 2016). These GOBP-branches can be found in detail, containing all module annotations in supplemental Table 2.

GOBP-Branch 1, named “organic substance metabolic process”, consists of metabolic processes such as DNA replication and repair, RNA translation and post-translational modifications. These metabolic processes underlie many other biological processes and are dysregulated in AD cases as a response to the various disease-related changes in the AD hippocampus (C.E. Humphries, et al., 2015,Liang, et al., 2008,Sekar, et al., 2015,Twine, et al., 2011,Wang, et al., 2016). The second GOBP-Branch, called “signal transduction”, consists of six gene modules that represent the same distinct neurotransmitter signaling pathways in both the discovery and replication datasets (all six gene module are in overlap category 1). These results indicate a broad dysfunction of synaptic transmission in the AD brain. These are likely the result of neuronal degeneration in AD hippocampus and are often found dysregulated in AD literature (Chi, et al., 2016,C.E. Humphries, et al., 2015,Liang, et al., 2008,Ray and Zhang, 2010,Sekar, et al., 2015,Wang, et al., 2016). GOBP-Branch 3, enriched for “transport”, mostly represents ion transport GOBP-terms, as shown in supplemental Figure 2. Many modules in this GOBP-branch are involved in energy production, which is often described as dysfunctional in previous AD studies (C.E. Humphries, et al., 2015,Wang, et al., 2016). These results are likely caused by neuronal degradation, and thus reduced energy consumption, although activation of glial cells might also influence this process (Sekar, et al., 2015). GOBP-Branch 4; “regulation of biological processes” is largely complementary to the other GOBP-branches. It contains modules annotated to both an executive biological process, e.g. “transmembrane transport” and its regulative process; “regulation of transmembrane transport”. Six of its seven gene modules fall into overlap category 1, indicating a robust dysfunction in this GOBP-Branch.

These first four GOBP-Branches are the largest and therefore underlie changes in AD pathophysiology that stand out the most in our study. Of the 33 identified gene modules in our study, 23 are involved in these four GOBP-Branches. With 17 gene modules in overlap category 1, this indicates that these four GOBP-Branches are amongst the most robust changes in AD pathophysiology. Given the functions of these central GOBP-Branches we conclude that organic substance metabolic processes, neurotransmitter signaling, energy transport and regulation of biological processes are main dysfunctional pathways in AD pathophysiology.

Of the remaining GOBP-Branch 5 (including RNA splicing and dephosphorylation) , Branch 6 (incl axon development) and Branch 7 (other metabolic processes), gene modules overlap mostly on in category 2 and category 3 with the replication dataset. These three GOBP-Branches do not contain any unique gene modules and are likely not as robustly involved in AD as the other GOBP-Branches. GOBP-Branch 8; "response to stimulus" is the smallest GOBP-Branch, indicating a response to neurodegeneration resulting in inflammation and glial cell activation which has often been observed in previous studies (C.E. Humphries, et al., 2015,Sekar, et al., 2015,Wang, et al., 2016). All three gene modules in GOBP-Branch 8 overlapped in category 1 with the replication dataset, suggesting that this small GOBP-Branch represents a robust change to AD pathophysiology. The biological processes of GOBP-Branch 9, including neuromuscular process", "actin-filament based movement" and "neuron projection guidance might also be robust changes in AD, but are represented by only a small number of gene modules in our data, possibly due to the late stage of the disease in our samples.

4.3. Interactions with AD genes

Of 27 AD genetic risk factor genes, only three were differentially expressed in our dataset, and two replicated (*MEF2C* and *PTK2B*). Several discovery modules interacted with these AD genes, suggesting a degree of overlap in biological function. *HLA-DRB1*, *HLA-DRB5*, *BIN1* and *PICALM* interact with M9 and might be involved in endocytosis and/or microtubule-based movement (Baig, et al., 2010,Zhou, et al., 2014). *ABCA7* and *MAPT* interact with a gene module involved in ion transport and signaling (M2). *APP* interacts with M1 and is involved in signal transduction (Cheng, et al., 2014,Cirrito, et al., 2008). *PTK2B* is differentially expressed in both discovery and replication and interacts with modules involved in receptor signaling and protein modification (M7, M10 and M25) (Beecham, et al., 2014,Han, et al., 2017). .*CELF1* interacts with genes involved in RNA processing and protein modification (M8) and *CLU* interacts genes involved in exocytosis and actin-based filament organization (M14). These interactions suggest roles of these genes also in later stages of AD, and do not represent the typical associations of these genes in a causal inference (Lambert, et al., 2013,Van Cauwenberghe, et al., 2016).

4.4. Limitations of this study

This study holds several limitations. Firstly, PPI networks are comprised of existing databases, which generate bias to well-known genes and biological processes (Gillis, et al., 2014,Schaefer, et al., 2015,von Mering, et al., 2003). Indeed, of the 2,716 DE-genes identified in discovery, only 1,610 held an interaction in the STRING database, and some relevant genes might have been excluded as a result.

An important issue in using PPI-data for your network analysis is that there are no clear guidelines on what to use for the interaction score cutoff, Markov clustering inflation factor threshold, or on the proper functional annotation of modules. Nevertheless some consensus is emerging and these most commonly used parameters were also applied in this study. These parameters are: 1. prioritizing or limiting to experimental interactions types, or not using text-mining based types, since this minimizes bias of the results (Szklarczyk, et al., 2017,von Mering, et al., 2003); 2. Optimizing the MCL inflation factor to generate modules of 10-100 genes (Subramanian, et al., 2005,van Dongen and Abreu-Goodger, 2012); 3. Replication, preferably on a functional annotation level as Gene Ontology (Ashburner, et al., 2000,Gene Ontology, 2015).

To optimize clustering of the gene modules, additional metrics of the generated PPI-network could be included, for example the direction of effect, or weighting PPI interactions. This study was designed as a cross-sectional case-control analysis, and many of the observed differences might be caused by neurodegeneration. Our sample size of 18 cases and 10 controls is not optimal to robustly detect all deviations in AD, and some genes/GOBP terms might have been missed.

4.5. Conclusions

Our method provide an comprehensive and complete overview of dysregulation based on GOBPs in AD. We show that the PPI and MCL clustering approach identifies functional gene modules which replicate in other datasets. Where individual genes might differ between studies, overall GOBP terms are preserved and can be

identified in this manner. Replication based on gene module GOBP terms was more robust than based on individual genes (82% versus 48%).

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FIGURES AND TABLES

Figure 1; flowchart of data analysis. Discovery and replication dataset are analyzed and differentially expressed genes are determined. A interaction network is constructed for each dataset, which is then clustered in gene modules. These modules are compared directly on overlapping genes and on enriched gene ontology biological processes. Interactions of modules identified in discovery with known AD disease genes are also investigated.

Figure 2; Volcano plot of 14,564 analyzed protein-coding genes. Each dot is a gene, those dark-grey pass the 0.1 DE score threshold. Upper score limits (set to maximum of 1) are displayed by dotted lines. The solid line displays the default FDR corrected ≥0.05 threshold. The Venn diagram displays the number of overlapping DE genes between the discovery and replication cohorts.

Figure 3; overlap between discovery and replication modules. Each module of discovery is shown horizontally, the replication modules are vertically. The numbers shown indicate the overlapping number of genes between two modules. Intersections marked in black indicate modules that share a gene ontology biological process. The last column indicates the category of overlap for each discovery module (1: overlap in genes and GOBP-term, 2: overlap in GOBP-term, but not in genes, 3: overlap in genes, but not in GOBP-term).

Table 1; Study sample characteristics. An asterisk denotes statistically significant difference compared to controls. All values represent means with standard deviations unless otherwise indicated. “Cases_QC” indicates metrics after removing two outlier cases.

Table 2; Overview of all three gene ontology biological processes of the discovery modules. Per module, the number of genes is shown. For each term, the name and respective GOBP branch is shown. The column “replication module” indicates which replication module also had this GOBP-term. The last column indicates interaction of discovery module with known AD disease genes.

Supplementary Figure 1; PC plot of discovery cohort, PC1 vs PC2.

Supplementary Figure 2; Overview tree of Gene Ontology Biological Processes per branch.

Supplemental Table 1; Expression matrix (14,564 genes, including test statistics, PPI statistics and module).

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Figure 1

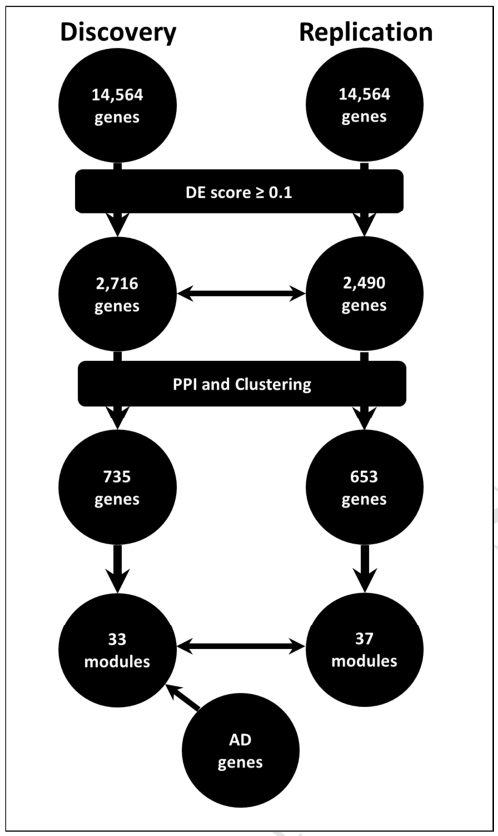

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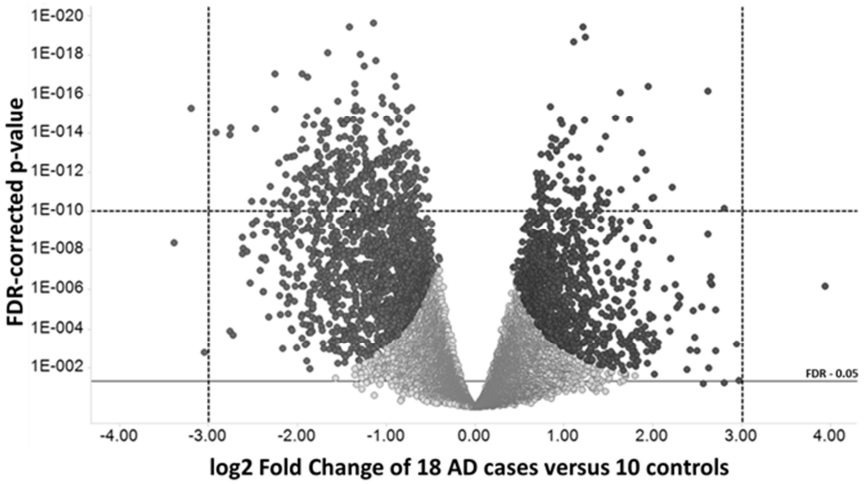
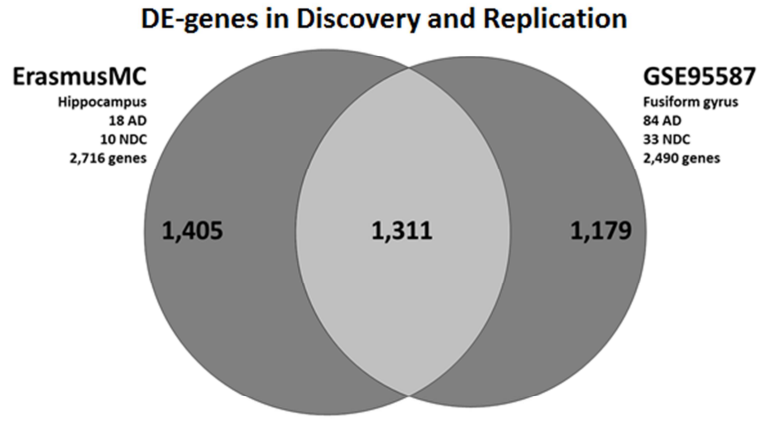
Figure 2





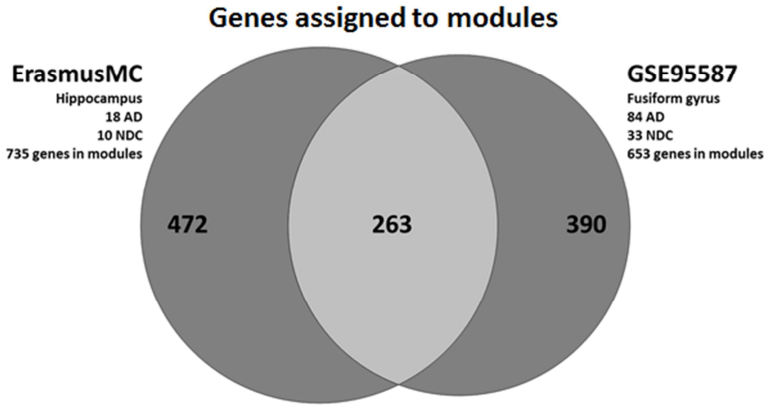


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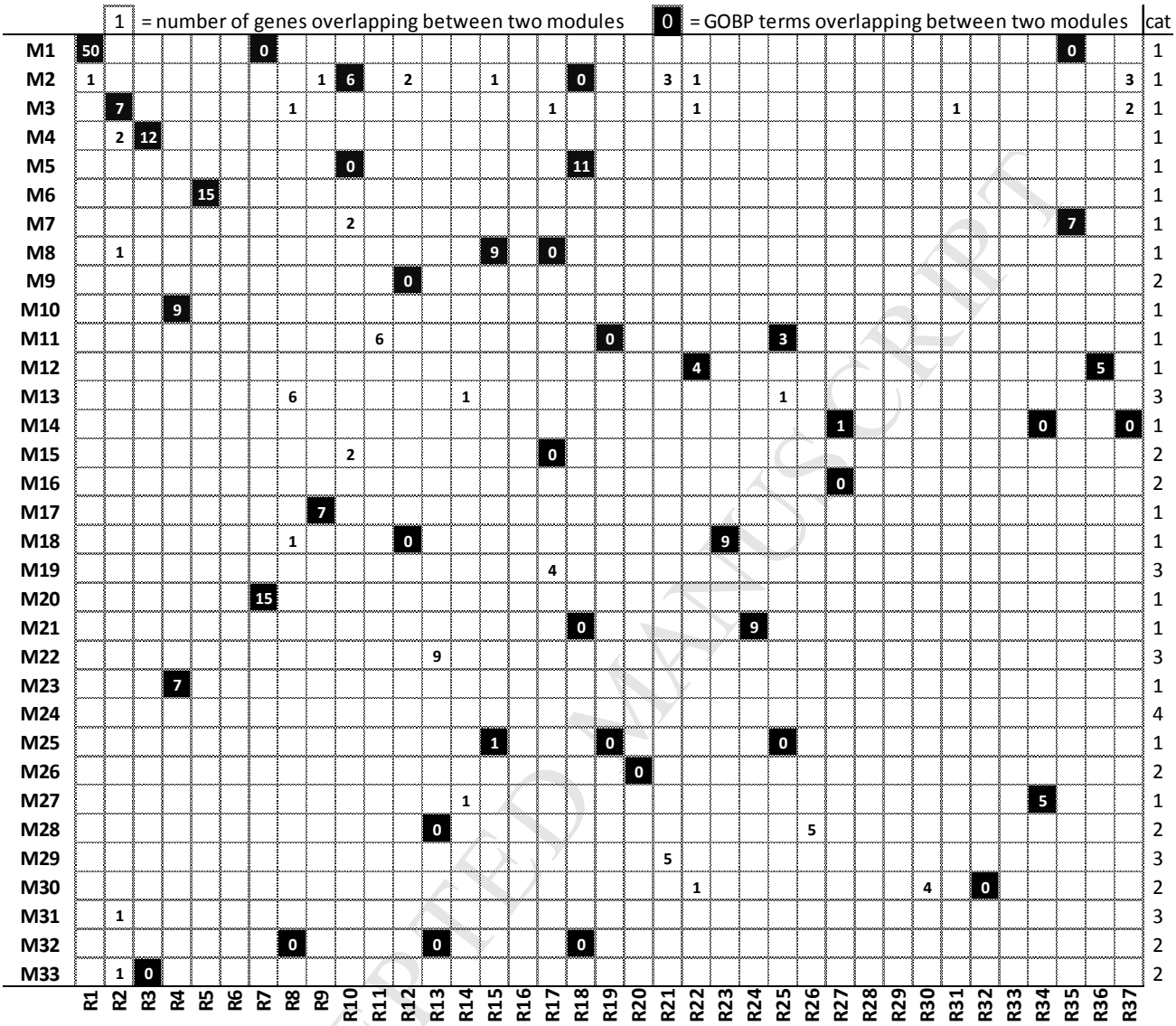
Figure 3



541 **Table 1**

	Controls	Cases	Cases_QC
Number	10	20	18
Gender (%Male)	50%	30%	44%
Age (±SD)	76 ± 12	75 ± 7	75 ± 7
Braak	1.5 ± 1.3	5.5 ± 0.5*	5.6 ± 0.5*
amyloid	0.9 ± 1.1	2.9 ± 0.3*	2.9 ± 0.3*
pmd	551 ± 297	348 ± 108*	329 ± 98*
pH	6.6 ± 0.3	6.3 ± 0.3*	6.3 ± 0.3*
brain weight	1319 ± 240	1045 ± 119*	1035 ± 113*
apoe (32/33/44)	4/6/0	1/9/10*	1/8/9*

542

Table 2

Discovery Module	Number of Genes	GOBP Branch	GOBP Term	Replication Module	AD genes
M1	90	2	G-protein coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	R1	APP
		2	phospholipase C-activating G-protein coupled receptor signaling pathway	R1	
		2	neuropeptide signaling pathway	R1, R7, R35	
M2	52	9	multicellular organismal signaling	R10	MAPT, ABCA7
		3	divalent inorganic cation transport	R10	
		4	regulation of transmembrane transport	R10, R18	
M3	39	1	DNA-templated transcription, initiation	R2	
		8	response to type I interferon	-	
		8	response to interferon-gamma	-	
M4	35	1	macromolecule deacylation	R3	
		1	histone modification	R3	
		4	regulation of chromatin organization	-	
M5	32	4	potassium ion transport	R18	
		4	regulation of transmembrane transport	R10, R18	
		6	protein oligomerization	R18	
M6	31	4	regulation of small GTPase mediated signal transduction	R5	
		2	Ras protein signal transduction	R5	
		4	regulation of cell morphogenesis	-	
M7	30	2	glutamate receptor signaling pathway	R35	PTK2B
		4	modulation of synaptic transmission	-	
		9	neuromuscular process	-	
M8	29	5	mRNA processing	R17	CELF1
		1	peptidyl-threonine modification	R15	
		5	RNA splicing	R17	
M9	27	3	receptor-mediated endocytosis	-	BIN1, HLA-DRB1, HLA-DRB5, PICALM
		9	microtubule-based movement	-	
		4	regulation of response to biotic stimulus	R12	
M10	26	2	integrin-mediated signaling pathway	R4	
		6	extracellular structure organization	R4	
		9	cell-substrate adhesion	-	
M11	21	1	peptidyl-tyrosine modification	R19, R25	
		6	axon development	-	
		1	ephrin receptor signaling pathway	R25	
M12	22	1	DNA replication	R36	
		1	DNA repair	R22, R36	
		1	DNA recombination	R22, R36	
M13	20	-	-	-	
		-	-	-	

		-	-	-	
M14	19	3	exocytosis	R27	CLU
		9	actin filament-based movement	R34	
		6	actin filament organization	R37	
M15	19	5	dephosphorylation	-	
		5	RNA splicing	R17	
		9	meiotic cell cycle	-	
M16	18	3	synaptic vesicle cycle	-	
		3	exocytosis	R27	
		3	neurotransmitter transport	-	
M17	17	8	stress-activated protein kinase signaling cascade	-	
		4	positive regulation of MAPK cascade	-	
		4	positive regulation of kinase activity	R9	
M18	18	2	I-kappaB kinase/NF-kappaB signaling	R12, R23	
		8	cellular response to biotic stimulus	R23	
		9	type I interferon production	-	
M19	17	1	translational elongation	-	
		1	mitochondrial translation	-	
		6	macromolecular complex disassembly	-	
M20	17	3	inorganic anion transport	R7	
		3	anion transmembrane transport	R7	
		2	gamma-aminobutyric acid signaling pathway	R7	
M21	15	3	transition metal ion transport	R24	
		3	hydrogen transport	R18	
		7	autophagy	-	
M22	15	6	NADH dehydrogenase complex assembly	-	
		6	mitochondrial respiratory chain complex assembly	-	
		9	mitochondrial respiratory chain complex I biogenesis	-	
M23	14	7	multicellular organism metabolic process	R4	
		6	extracellular structure organization	R4	
		-	-	-	
M24	13	7	glycerolipid metabolic process	-	
		7	lipid modification	-	
		5	phospholipid metabolic process	-	
M25	13	1	peptidyl-serine modification	R15, R19, R25	
		-	-	-	
		-	-	-	
M26	12	1	protein ubiquitination involved in ubiquitin-dependent protein catabolic process	-	
		1	protein polyubiquitination	-	
		7	amine metabolic process	R20	
M27	12	9	neuron projection guidance	R34	
		-	-	-	

		-	-	-
M28	11	5	organophosphate catabolic process	-
		1	carbohydrate derivative catabolic process	R13
		5	aromatic compound catabolic process	-
M29	11	-	-	-
		-	-	-
		-	-	-
M30	10	5	pyruvate metabolic process	R32
		7	small molecule catabolic process	-
		7	carbohydrate catabolic process	-
M31	10	-	-	-
		-	-	-
		-	-	-
M32	10	3	mitochondrial transport	R8
		5	nucleoside monophosphate metabolic process	R13
		3	hydrogen transport	R18
M33	10	6	chromatin remodeling	R3
		9	protein acylation	R3
		-	-	-

Highlights (Mandatory, submitted as separate file, max 85 characters per bullet, 3-5 bullets)

- 2,716 differentially expressed genes in 18 AD hippocampus compared to 10 controls
- 33 differentially expressed gene modules identified by PPI network clustering
- 48% of genes replicate vs 82% of annotated GOBP-terms
- Gene modules represent specific subsets of enriched biological processes