Microarray Analysis on Human Neuroblastoma Cells Exposed to Aluminum, β_{1–42}-Amyloid or the β_{1–42}-Amyloid Aluminum Complex

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Abstract

Background: A typical pathological feature of Alzheimer’s disease (AD) is the appearance in the brain of senile plaques made up of β-amyloid (Aβ) and neurofibrillary tangles. AD is also associated with an abnormal accumulation of some metal ions, and we have recently shown that one of these, aluminum (Al), plays a relevant role in affecting Aβ aggregation and neurotoxicity.

Methodology: In this study, employing a microarray analysis of 35,129 genes, we investigated the effects induced by the exposure to the Aβ_{1–42}-Al (Aβ-Al) complex on the gene expression profile of the neuronal-like cell line, SH-SY5Y.

Principal Findings: The microarray assay indicated that, compared to Aβ or Al alone, exposure to Aβ-Al complex produced selective changes in gene expression. Some of the genes selectively over or underexpressed are directly related to AD. A further evaluation performed with Ingenuity Pathway analysis revealed that these genes are nodes of networks and pathways that are involved in the modulation of Ca^{2+} homeostasis as well as in the regulation of glutamatergic transmission and synaptic plasticity.

Conclusions and Significance: Aβ-Al appears to be largely involved in the molecular machinery that regulates neuronal as well as synaptic dysfunction and loss. Aβ-Al seems critical in modulating key AD-related pathways such as glutamatergic transmission, Ca^{2+} homeostasis, oxidative stress, inflammation, and neuronal apoptosis.

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Introduction

The abnormal deposition and aggregation of β-amyloid (Aβ) in senile plaques are hallmarks features of the Alzheimer’s disease (AD) brain. Senile plaques are made up of aggregates of misfolded Aβ that are associated with high concentrations of several endogenous or exogenous metal ions (Fe, Zn, Cu, and Al), but they also contain cell elements migrating from the immune-response system [1,2]. Metal ions have been indicated as important co-risk factors in several neurodegenerative disorders [3,4] and, in the context of AD, recent studies have shown that they are key in accelerating Aβ oligomerization as well as modifying the neurotoxic properties of the amyloid peptide [5,6,7]. In that respect, we have recently shown that Fe, Zn, Cu, and Al each can specifically affect the pathogenic actions of Aβ [8]. We have also reported that, compared to other Aβ-metal complexes (Aβ-Fc, Aβ-Zn, Aβ-Cu), Aβ-Al is unique in promoting a specific form of Aβ oligomerization that has marked neurotoxic effects [8].

In this study, we have continued to explore the molecular determinants involved in the toxicity induced by the Aβ_{1–42}-Al (Aβ-Al) complex. To that aim, we investigated changes in the gene expression profile of neuronal cell lines, the SH-SY5Y, exposed to either Aβ, the Aβ-Al complex or Al alone, against untreated cultures. SH-SY5Y cells were chosen because as cell line they offer the advantage of being a homogenous population that does not show the subtype heterogeneity present in primary neuronal cultures, a confounding factor that would make the results hard to interpreter.

Cultures were exposed to Aβ, the Aβ-Al complex or Al alone and investigated for gene profile changes by microarray analysis, a
Gene Expression of SH-SY5Y Cells Exposed to Aβ-Al

Networks associated with genes selectively changed by the exposure to the Aβ-Al complex

IPA predicts functional networks based on known protein-protein and functional interactions. IPA infers and ranks networks by a score, expressed as a numerical value, which is a probabilistic fit between the amount of focus genes that are potentially eligible for a network composition and present on a given gene list, the size of the network, as well as all the molecules present in the Ingenuity Knowledge Base that can be part of such a network. We therefore employed IPA to study how the genes selectively changed in their expression by the Aβ-Al exposure were interacting in specific networks.

Networks associated with upregulated genes. Analysis of the upregulated genes indicated 25 networks with a score ranging from 46 to 13, the four top networks generated by IPA are provided in Fig. 2 and Tables S3, S4, S5, S6.

The first network (Fig. 2A, Table S3) involves nodes of genes that participate in the inflammatory response (TGBF1) as well as in synaptic functioning (ARRB1 and APLP1).

The second network (Fig. 2B, Table S4) involves nodes of genes that play an important role in oxidative stress (NOS), endocytosis (clathrin), inflammatory response (AP1), and apoptosis (JUN).

The third network (Fig. 2C and Table S5) involves nodes of genes that serve a role in AβPP processing (Furin), synapse development (MEF2), neuroprotection (NR41), insulin metabolism (IDE), and histones deacetylation (HDAC9).

The fourth network (Fig. 2D and Table S6) is built on nodes of genes that participate in the cleavage of fatty acids (PLA2), in calcium signalling (calmodulin) as well as in glutamatergic neurotransmission (GRIN1, GRIN2c).

Networks associated with downregulated genes. Analysis of the downregulated genes showed 25 networks with a score ranging from 39 to 14. The four top networks are provided in Figure 3 and Tables S7, S8, S9, S10.

The first network (Fig. 3A and Table S7) is centered on nodes of genes involved in the morphological and functional integrity of synapses (Homer, Homer I), preservation of cholinergic activity (ESR1), and gene expression (EP300). The second network (Fig. 3B and Table S8) shows nodes involved in apoptosis (CDH1) and cell motility (G-actin).

The third highest ranked network (Fig. 3C and Table S9) is focused on down regulated genes that are involved in biological functions related to aminoacid metabolism and post translational modification. The main network nodes are represented by Protein kinase C (PKC) and SHANK2.

The fourth down regulated network (Fig. 3D; Table S10) is centered on nodes of genes that are playing a role in the inflammatory response (CD3) and oxidative stress (GST).

Pathways associated with genes selectively changed by the exposure to the Aβ-Al complex

We performed a pathway analysis of the genes selectively modulated by the exposure to the Aβ-Al complex. This analysis reveals two main pathways that are likely involved in AD.
The first pathway pertains to the regulation of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) homeostasis, a mechanism that has functional consequences on neuronal and dendritic/spine loss as well as in the maintaining of Long-term Potentiation (LTP) and memory (Figure 4). This pathway shows the upregulation of genes encoding for key proteins like glutamate receptor subunits (GRIN1, GRIN2c, and GRIA), CAMKII, HDAC, MEF2, Actin alfa, as well as NCX, the gene encoding for the Na\(^+\)/Ca\(^{2+}\)-exchanger, PMCA that encodes for the Na\(^+\)/Ca\(^{2+}\)-ATPase CRACs are critical channels controlling capacitative Ca\(^{2+}\) entry and the maintaining of Ca\(^{2+}\) homeostasis when the cation is depleted from intracellular stores [19].

Finally, we find a downregulation of CRAC that encodes for activation of store-operated Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels. CRACs are critical channels controlling capacitative Ca\(^{2+}\) entry and the maintaining of Ca\(^{2+}\) homeostasis when the cation is depleted from intracellular stores [19].

The second pathway inferred by IPA is functionally related to the first one and shows genes that are controlling glutamatergic neurotransmission. In the second pathway we find upregulated genes encoding for NMDA and AMPA receptor subunits (GRIN, GRIA and GRID) as well as downregulated genes, like HOMER, calmodulin. Downregulated are also SLC1A 6/7 and SLC1A 2/3, that encode for glutamate transporters as well as SLC1A 2/3, that encode for glutamate transporters

**Table 1.** List of AD-related genes (http://www.alzgene.org) that are selectively overexpressed upon exposure to A\(\beta\)-Al compared to exposures to A\(\beta\) or Al alone.

| Gene symbol | A\(\beta\) (Log\(_2\) ratio) | A\(\beta\)-Al (Log\(_2\) ratio) | Al (Log\(_2\) ratio) | RefSeq | Description |
|-------------|-----------------|-----------------|-----------------|--------|-------------|
| AR          | −0.15           | 1.94            | 0.42            | NM_000044 | Androgen receptor |
| NOS2A       | −0.21           | 1.87            | −0.14           | NM_006255 | Nitric oxide synthase, inducible (NOS, type II) |
| VDR         | 0.19            | 1.77            | 0.17            | NM_000376 | Vitamin D3 receptor (VDR) |
| NGFR        | −0.12           | 1.18            | −1.12           | NM_002507 | Tumor necrosis factor receptor |
| CCNT1       | 0.28            | 1.09            | −0.06           | NM_001240 | Cyclin T1 |
| IDE         | −0.02           | 1.05            | −0.16           | NM_004969 | Insulin-degrading enzyme |
| FTSJ3       | 0.13            | 1.02            | 0.41            | NM_017647 | FtsJ homolog 3 |
| KCNJ6       | −0.53           | 1.02            | 0.36            | NM_002240 | G protein-activated inward rectifier potassium channel 2 |
| NOS1        | 0.43            | 1.01            | −0.74           | NM_006620 | Nitric-oxide synthase, brain (NOS, type I) |
| PLCE1       | 0.27            | 0.99            | 0.34            | NM_016341 | pancreas-enriched phospholipase C |
| PRIKA1      | −0.02           | 0.93            | 0.09            | - | 5‘-AMP-activated protein kinase, catalytic alpha-1 chain |
| SLC6A3      | 0.22            | 0.90            | 0.35            | NM_001044 | Sodium-dependent dopamine transporter |
| MYH8        | 0.38            | 0.85            | −0.21           | NM_002472 | Myosin heavy chain, skeletal muscle, perinatal |
| FDP5        | −0.22           | 0.84            | 0.13            | NM_002004 | Farnesyl pyrophosphate synthetase |
| HMOX1       | 0.15            | 0.83            | 0.30            | NM_002133 | Heme oxygenase 1 |
| CNTF        | 0.22            | 0.82            | 0.03            | NM_170768 | Ciliary neurotrophic factor |
| SOS2        | 0.32            | 0.79            | −0.69           | NM_006939 | Son of sevenless protein homolog 2 |
| ACSL4       | 0.40            | 0.74            | −0.02           | NM_00458 | Long-chain-fatty-acid-CoA ligase 4 |
| ADRB1       | 0.35            | 0.73            | −0.28           | NM_00684 | Beta-1 adrenergic receptor |
| ABCA1       | 0.05            | 0.72            | 0.45            | - | ATP-binding cassette, sub-family A, member 1 |
| APOM        | 0.00            | 0.59            | −0.63           | NM_019101 | Apolipoprotein M |
| GLP1R       | 0.36            | 0.55            | 0.21            | NM_002062 | Glucagon-like peptide 1 receptor precursor |
| CH25H       | −0.14           | 0.55            | −0.47           | NM_003956 | cholesterol 25-hydroxylase |
| ECE1        | −0.06           | 0.51            | −0.07           | NM_001397 | Endothelin-converting enzyme 1 |
| ATF7        | −0.07           | 0.51            | −0.04           | NM_006856 | Cyclic-AMP-dependent transcription factor |
| F13A1       | 0.30            | 0.50            | 0.22            | - | Coagulation factor XIII A chain precursor |

The involvement of these pathways in AD pathogenesis is further analyzed below in the discussion.

**Discussion**

The IPA-generated network and pathway analysis reveals that, compared to A\(\beta\) or Al alone, the A\(\beta\)-Al complex selectively...
modulates the expression of genes that can have an important role in AD pathogenesis.

For clarity, we have broken down the discussion on specific genes, analysis of networks, and pathways.

Gene analysis

**Upregulated genes of the first network.** TGFβ1 encodes for the Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 protein that forms a heteromeric complex with the TGF-beta receptors type II and participates in the transforming growth factor (TGF)-1 signaling cascade. TGF Beta -1 can have a role in AD as experiments in AD transgenic mice have indicated that the protein promotes a marked reduction in brain accumulation of AβPP through the activation of microglia [20]. Thus, TGFβ1 overexpression may promote a protective compensatory inflammatory response that can reduce the brain amyloid load.

ARRB1 belongs to the Beta-arrestin family and modulates the desensitization and endocytosis of seven-transmembrane receptors, 7TMRs, the largest group of plasma membrane receptors [21]. In the context of AD, ARRB1 may be critically important as it can modulate the endocytosis of metabotropic glutamate receptors, mGlRs, and affect glutamatergic neurotransmission [22]. Furthermore, ARRB1 can interact with APLP1 in promoting neuronal apoptosis [21].

APLP1 is a member of a protein family that also includes the Aβ precursor protein (AβPP). APLP1 encodes for a membrane-associated glycoprotein that, like AβPP, is cleaved by secretases. While AβPP has received much attention in the context of AD as the protein is involved in neurite outgrowth, cell adhesion, synaptogenesis, synaptic plasticity and neuroprotection [23], far less studies have investigated the role of APLPs. Despite a high degree of similarity with AβPP, APLP1 lacks an amyloidogenic domain; however, APLP1 has been found in the amyloid plaques of AD brains [24,25,26]. Furthermore, given its synaptic localization, APLP1, like AβPP, can be involved in synaptogenesis and synaptic plasticity. APLP1 also influences AβPP endocytosis [27] and an accumulation of APLP1 has been reported in neuritic plaques of the hippocampus of AD patients [24].

**Upregulated genes of the second network.** Nitric oxide synthase (NOS) generates nitric oxide (NO) and is present in the CNS in three isoforms: neuronal Type-1 NOS (nNOS), inducible Type-2 NOS (iNOS), and endothelial Type-3 NOS (eNOS). iNOS is induced in astrocytes near the senile plaques [28] and, in transgenic AD animal models, its overexpression is an early event that precedes the appearance of amyloid plaques [29]. An increased NOS-mediated NO production can promote neuronal dysfunction and/or cell death. NO is also a major determinant of NMDAR-dependent neurotoxicity and, as such, can be a key player in the excitotoxic neuronal loss found in AD [30,31]. Cliahrin-is a key protein that regulates endocytosis. In the context of AD, several studies indicate that the endocytotic machinery plays an active role in AβPP [32]. Furthermore, clathrin-mediated endocytosis is also a regulator of the internalization of a subtype of ionotropic glutamate receptor, the AMPA receptor (AMPAR),

### Table 2. List of AD-related genes (http://www.alzgene.org) that are selectively downexpressed upon exposure to Aβ-Al compared to exposures to Aβ or Al alone.

| Gene Symbol | Aβ (Log2 ratio) | Aβ-Al (Log2 ratio) | Al (Log2 ratio) | RefSeq | Description |
|-------------|-----------------|--------------------|----------------|--------|-------------|
| OLIG2       | −0.09           | −1.93              | 0.68           | NM_005086 | Oligodendrocyte transcription factor 2 |
| NAT1        | −0.39           | −1.68              | −0.24          | NM_000662 | Arylamine N-acetyltransferase 1 |
| APOC3       | 0.67            | −1.00              | −0.35          | NM_000040 | Apolipoprotein C-III precursor |
| WNT8B       | 0.54            | −0.96              | 0.68           | NM_003393 | Wnt-8b protein precursor |
| MMSE        | −0.20           | −0.93              | −0.26          | NM_007289 | Neprilysin |
| APOD        | 0.36            | −0.88              | −0.08          | NM_001647 | Apolipoprotein D precursor |
| LRP1        | −0.13           | −0.84              | −0.35          | NM_002332 | Low-density lipoprotein receptor-related protein 1 precursor |
| AHSG        | −0.49           | −0.78              | −0.47          | NM_001622 | Alpha-2-HS-glycoprotein precursor |
| GAB2        | −0.13           | −0.78              | −0.05          | NM_080491 | GRB2-associated binding protein 2 |
| MMP1        | −0.20           | −0.73              | 0.27           | NM_002421 | Interstitial collagenase precursor |
| TCN1        | 0.25            | −0.71              | −0.05          | NM_001062 | Transcobalamin I precursor |
| MYST4       | 0.18            | −0.68              | 0.15           | NM_012330 | MYST histone acetyltransferase |
| TNFRSF1B    | 1.06            | −0.67              | 0.16           | NM_001066 | Tumor necrosis factor receptor superfamily member 1B precursor |
| ANXA8       | 0.28            | −0.63              | 0.22           | -       | Annexin A8 |
| CD36        | 0.36            | −0.61              | 0.35           | NM_001001548 | Platelet glycoprotein IV |
| CTSS        | −0.36           | −0.60              | 1.25           | NM_004079 | Cathepsin S precursor |
| ESR1        | 1.01            | −0.60              | −0.26          | NM_000125 | Estrogen receptor |
| SFRP5       | 0.43            | −0.58              | 0.09           | NM_003015 | secreted frizzled-related protein 5 |
| PIK3R1      | −0.43           | −0.56              | −0.50          | NM_181524 | Phosphatidylinositol 3-kinase regulatory alpha subunit |
| POU2F1      | 0.32            | −0.54              | 0.34           | NM_002697 | POU domain, class 2, transcription factor 1 |
| TNF         | 0.01            | −0.52              | 0.39           | NM_000594 | Tumor necrosis factor precursor (TNF-alpha) |
| THRA        | −0.48           | −0.52              | −0.35          | NM_003250 | Thyroid hormone receptor alpha |
| FCER1G       | −0.26           | −0.52              | −0.01          | NM_004106 | High affinity immunoglobulin epsilon receptor gamma-subunit precursor |

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that critically modulates the efficacy of glutamatergic neurotransmission as well as LTP and Long-term Depression [33]. Modulation of synaptic activity impinges in amyloid metabolism as sustained synaptic activation leads to a rapid increase in Aβ levels in brain interstitial fluid (ISF) while a depressed synaptic transmission reduces Aβ ISF levels [34].

C-Jun is a component of the AP-1 protein complex and AP-1 is activated downstream of c-Jun [35]. The c-Jun N-terminal Kinase (JNK)-AP1 signaling pathway plays a key role in AD by affecting gene expression, cell proliferation, the inflammatory response as well as neuronal apoptosis [36]. In AD brains, c-Jun phosphorylation promotes the induction of JNK activity, a phenomenon

Figure 1. Biological functions associated with genes selectively changed by the exposure to the Aβ-Al complex. Bar charts show key biological functions associated with genes found to be selectively either overexpressed (A) or downexpressed (B) in SH-SYSY cells exposed to Aβ-Al. doi:10.1371/journal.pone.0015965.g001
the development of late onset AD [50,51]. Finally, IDE has also been found to be decreased upon aging and AD [52].

Histone deacetylases (HDACs) belong to a family of proteins that by catalyzing the deacetylation of histones plays an important role the epigenetic brain regulation of transcription, apoptosis as well as learning and memory [53,54]. Interestingly, many neuroprotective genes such as BDNF, GDNF, HSP70, α-B-crystallin, Bcl-2, Bcl-XL, and p21 are modulated by HDAC [37,38]. HDACs are also involved in the development of late onset AD [39,40]. Interestingly, furin mRNA levels have been shown to be reduced in the brain of AD patients and transgenic AD mice [41].

Upregulated genes of the third network. Furin participates in the amyloid metabolism by regulating the cleavage of AβPP through α-secretase activation [38], thereby reducing Aβ production. The process is mediated by promoting the maturation of A Disintegrin And Metalloproteases 10 (ADAM10) and ADAM 17/tumor necrosis factor-α converting enzyme (TACE), two enzymes that have α-secretase activity [39,40]. Interestingly, furin mRNA levels have been shown to be reduced in the brain of AD patients and transgenic AD mice [41].

The Myocyte enhancer factor 2 (MEF2) is a Ca2+-regulated transcription factor that promotes neuronal survival [42] and is also a key modulator of activity-dependent synaptic development [43]. MEF2C is a transcription factor that facilitates hippocampal-dependent learning and memory in rodents. MEF2C is positively modulated by BDNF [44] and regulates the synaptic number as well the efficacy of synaptic connections [45].

NR41 is a member of a pool of 9 neuroprotective genes called Activity-regulated Inhibitor of Death (AID) genes that have been shown to promote neuronal survival by making neuronal mitochondria more resistant to cellular stress [46].

Table 3. Central nervous system (CNS) genes present in the functional groups associated with overexpression promoted by the exposure to the Aβ-Al complex.

| Category                        | p-value | Molecules                                                                 |
|---------------------------------|---------|---------------------------------------------------------------------------|
| Amino Acid Metabolism           | 2.23E-04| NS05, SLCE6A8, TGFB1R1, ATG7, TDO2, HIPK3, BHMT, SLCA13, NAGS, IDO2, SLCA7A, BDKBR2, FTCD, SBK1, SLCA16A, GNRH1, NO52, SLCA7A, ARG1 |
| DNA Replication, Recombination, Repair | 5.41E-04 | NS01, BDKBR2, DMC1, NO52, NUDT15                                           |
| Lipid Metabolism                | 5.57E-04 | PTG5, RAB27B, PLA2G3, TNSF510, ARSA, CES1 (includes EG:1066), PLCD1, BDKBR2, HMOX1, F2RL3, AR, PLC1, ARRB1, INPP5F, PLA2G2D, ACNP, NERF, GPD1, GNRH1, KISS1, VDR, NO52, LPP |
| Cellular Function and Maintenance | 1.19E-03 | NS01, SPIB, C3, STX6, SRL, CD33, JUN, CAMK2A, CBL, AR, CXCL13, NGFR, S1PR1, GNRH1, KIT, PRDM1, VDR, FASLG |
| Cell Death                      | 1.49E-03 | NS01, FURIN, TGFB1R1, ATF7, NEFL, TNSF510, HMOX1, YES1, JUN, AR, PDCD1, NGFR, KIT, PRIR1, PRIS3, NO52, FASLG, EBAG9, ITGA2, SLCA8A, F13A1, MLANA, CLCF1, HMGA2, CTF1, NRAA1, HAS2, RARRES3, ARG1 |
| Cell Morphology                 | 1.49E-03 | NS01, IFT88, KALRN, LMO2, RP1, PROX1, PKRG1, LAMB2, BDKBR2, AR, ESPN, NGFR, EFNB1, EFNAS, TNCSAP1, VDR, CALML3, MARCD, ARG1 |
| Cell Signaling                  | 1.49E-03 | NS01, DPP3, STX6, F13A1, TNSF510, KNCIP3, IL12R2B, BDKBR2, HMOX1, CAMK2A, JUN, S1PR1, VDR, NO52, FASLG, ATP2B4, ARG1 |
| Nervous System Development and Function | 1.49E-03 | NS01, POUS3F1, NODAL, SLCA13, EMX2, PKRG1, EN1, CAMK2A, AR, ABR, APLP1, EFNAS, NGFR, MAL, CDSK2R, NHH2L, ECE1, TNCSAP1, NO52, IRX3, ZBTB16, FRS2, IFT88, KALRN, ARSA, CLCF1, LAMB2, SEPP1, PYG02, CTF1, ARHGEF10, GNRH1, S1PR1, PTH2, ARG1, ATDH7 |
| Cell Compromise                 | 1.79E-03 | NS01, C3, SLCA13A, TNSF510, PKRG1, LINM1, BDKBR2, HMOX1, JUN, NGFR, EFNAS, EFNB1, GNRH1, KIT, CEP250, TRRAP |
| Neurological Disease            | 5.25E-03 | SNTG1, KIF13B, FURIN, CACNA1H, NKAIN3, IDE, MYH7B (includes EG:57644), TESC, ILDR1, CAMK2A, KIF13A, APLP1, SLCA16A2, H656HT (includes EG:26672), HIPK1, ZBTB16, FRS2, PTG5, SLCAAD4, CYP7B1, ATG7, APOM, LKRNH, LPHN3, GPR15, IL12R2B, ZNF215, MED12, AIG1, RASL12, ARHGDIB, NNXV1, GPCS, SRGAP1, PDBXL2, PDSK1, SCN1B, ST3GL4I, EHTM2, NE4, CXCR6 (includes EG:10663), HAS2, CYCS (includes EG:54205), NR3C2, RCVN1, TSNARE1, FLJ45664, ITGA2B (includes EG:3674), UBA3SHA, SCD5, MYH8, HLA-DQA1, SLCA13A, HSPB8, TNSF510, TME185A5, HTR1D, S1PR1, PKRG1, CORO2B, TYW1, SLCA63, MAFG, PLCD1, CNGL1, YES1, PRXK, AR, ARB1, ADRB1, JUN, TRHDE, GRID1, NGFR, EFNAS, MAL, ACSL4, LLY5, DEG52, EF2K, VDR, MUC2, LPP, TRIM67, DNER, PFKT, CDDCE, CXXL6, TAA66, CXYRF4A8, SVIL, GLE1, GLT2ED2, NR4A1, S1PR1, RXBPAG2, COL44A4 (includes EG:12686), AK3L1, ACVR2A, STSBS1A3, STRN, T, CLTB, EDA, TRIM31, USH1C, LIMK1, SHC4, GGAG1, ESPN, GRIN2C, BSRK1, SPPL2, C2D9, NUPR1, TNCSAP1, ACT1, IFT88, NDUF53, SEC14L3, C3, SESN3, PSMF1, MAST4, MYH14, ARID1B, COL2A1, CDC129, ARSA, CHRND, SLCA61A, CYPOR57, SLCC2A5, GNRH1, RARRES3, RXFK2, CA11, KCN6, C1ORF21, CNHA, SYNPR, AGTR1, URRCSB, ARG1, NO51, OU3F1, CCIN, NEFL, GPR156, FLRT2, PCDH91, EMX2, EOXN1, KIAA1409, ZNF225, OPR1, HMOX1, PLA2G4E, FXD770, LGRK3, JUN, NAMPT, NO52, LSP2, CNP1, CEOR6, GORF51, BTLA, RRGRP1, WDR25, SCN1A, HLA-DMA, COLS5A2, DSPS, MDGAD1, RPI1, SLCA83, TRPA1, F13A1, CCDC85A, KNCIP1, CCL4, PLXND1, AFF1, FDS, SEPP1, ARHGEF10, DIP2C, HERC6, AKAP10, FOX1, BCA5I, GRIA3 |

RNA Damage and Repair 3.86E-02-3.86E-02 NS02

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inhibition. Furthermore, several findings indicate that HDAC inhibition in AD animal models restores the histone hypoacetylation, increases synaptic plasticity, decreases Aβ production and tau hyperphosphorylation, improves learning and memory and reverses spatial memory deficits [55,56]. Finally, experimental data show that overexpression of HDAC9 and HDRP, a spliced form of HDAC9, can serve a role as an antiapoptotic molecule by binding and inhibiting the apoptosis-inducing MAP kinase, JNK [57].

Upregulated genes of the fourth network. Phospholipase A2 (PLA2) participates in the metabolism of fatty acids [58,59]. PLA2 modulates several signaling pathways that link oxidative stress and proinflammatory cytokines to the release of arachidonic acid and the synthesis of eicosanoids. PLA2 is also involved in intracellular membrane trafficking, differentiation, proliferation, and apoptosis [60]. The role of PLA2 in AD is complex. Reduced activity of specific subtypes of intracellular PLA2 (cPLA2 and iPLA2) are found in AD and thought to participate in the cognitive decline and neuronal loss associated with the disease [61]. On the other hand, PLA2 has been found to be strongly overexpressed in AD, suggesting that, overall, this gene may have a negative effect by promoting the inflammatory response of the AD brain [62].

The Calcium/calmodulin-dependent protein kinase II, CaM kinase II, is the most abundant protein kinase in the brain and by controlling the trafficking of AMPARs plays a major role in synaptic plasticity, which is impaired in AD [63]. A key kinase II, is the most abundant protein kinase in the brain and by promoting the inflammatory response of the AD brain [62].

Table 4. Central nervous system (CNS) genes present in the functional groups associated with downexpression promoted by the exposure to the Aβ-Al complex.

| Category                        | p-value | Molecules                                                                 |
|---------------------------------|---------|---------------------------------------------------------------------------|
| Cell-To-Cell Signaling and Interaction | 1.44E-06-2.05E-02 | TAF4B, GABZ, CD3E, TGFB3, POU2AF1, PIK3R1, LAT2, GHSR, FOXP3, CD1E, CD163, TREM2, STAB2, F2, PTPRC, HRH1, ALDH1A1, HAVCR1, PIK3CG, PPBP, TAs1R3, CD26, OSM, KLRC3, SCNSA, PTX3, KIR2D05, GLRA2, OPRM1, CD200R1, SNA1, OPMCL, PECLE, TRELC, FYB, LAX1, TLR2, TNFSF8, PLNLPPR2, CD1H, PGLYRP4, UMOD, GJ3, IL10RA, PECAM1, BCL2A1, TNF, LCP2, TCLA1, ALK, ADM, MSR1, PLA2G10, KLRF1, ADIPQ, RH01, WNT7A, CSAR1, PDCD1L2,APC2, IL2, TAOK2, CD36, PRCKE, SL100A8, IL2RA79A, TNFRSF18, CFP, MMP1, RNAS2, ALOX5, BCL2A1, GHI, CD200R1, PLEC, TREM2, CD66, IFNA6, F2, STAB2, MTPP, HAVCR1, CD226, PIR13, OSM, MGM, SCNSA, PTX3, OPRM1, CLEC1A, LAMDA2, THRA, PDE4B, HHP, FYB, LAX1, MECOM, TLR2, PGLYRP4, UMOD, WDFC21C, PECAM1, RAG1, IL2RA2, ESRI, TCLA1, MSR1, AMBP, PDCD1L2, LYST, IL2, PRAKR1B, S100A8, RELB, CD36, MGAT5, IL1R1, IL20RB, APO1L, CCL7, RXL12, PISL, SL1C2A2, NFTAC2B,  |
| Inflammatory Response | 5.08E-06-2.12E-02 | TAF3, PKRF3, CD1E, AFAP1L2, PTTPRC, HRH1, CTSS, PIK3CG, PPBP, ALPP, ALPR2, ZAB27A, CD200R1, PLEC, CLESCA, CDH1, HLA-DRB1, TNF, LCP2, MME, AD, PLA2G10, KLRF1, ADIPQ, FOXN1, TRMP2, NIC3, CSAO1, TAOK2, CD36, PRCKE, IL27RA, TNFRSF18, CFP, MMP1, RNAS2, ALOX5, BCL2A1, CD200R1, PLEC, CLESCA, CDH1, IL1R1, L26R, TNF, LCP2, CA2, ADIPAQ, TWS51, FOXN1, EP300, CLEC11A, CSAR1, WNT7A, CD38, IL28RA, TNFRSF18, MMP1, RNAS2, ALOX5, UCH51, PHAUPF, CACNB4, TAF8, GNAQ, HIP1, ID3, SPDFA, BCL1, FCER1G, TIRAP, CHRD1, PTTPR2, INF1A1, POFU1, PTIRK, TC21, TAF4B, GAB2, CD3E, POU2AF1, C4LA, SKL2, TAF8, FXP3, RNAS1, EDME1, CD163, TREM2, MEN1, CD88, IFNA6, F2HUS, MBNL3, ALDH1A1, HAVCR1, CD226, MRA5, OSM, FGL, 2MYM2, TXB19, LAMDA2, THRA, CITED1, LAX1, MLPH, MECCOM, TRL2, GJ3, RAG1, PECAM1, SF3B4, ESRI, TCLA1, FGF5, GATA1, MSR1, PICALM, CHRD2L2, RHOD, BLK, IL2, GNA72, RELB, EMXI, CD36, ERBB3, HE35, IL1R1, NKX2-5, CXL12, LING01, SL1C2A2, NFTAC2B |
Figure 2. Top networks generated by IPA associated with genes selectively overexpressed by the exposure to the Aβ-Al complex. Genes in red show increased expression in SH-SY5Y cells exposed to Aβ-Al when compared with untreated SH-SY5Y cells. Arrows indicate that a molecule acts on another while lines indicate that a molecule binds to another. Small histograms indicate changes in gene expression. doi:10.1371/journal.pone.0015965.g002
Figure 3. Top networks generated by IPA associated with genes selectively downexpressed by the exposure to the Aβ-Al complex. Genes in green show decreased expression in SH-SY5Y cells exposed to Aβ-Al when compared with untreated SY5Y cells. Arrows indicate that a molecule acts on another while lines indicate that a molecule binds to another. Small histograms indicate changes in gene expression. doi:10.1371/journal.pone.0015965.g003
receptor subunits, IPA shows a very intriguing upregulation of two specific subunits, GRIN1 and GRIN2c, and the functional implications of such changes are discussed in the pathway section.

**Downregulated genes of the first network.** Homer family is a class of postsynaptic scaffolding proteins that regulate the structural and functional integrity of synapses [66]. Homer I is particularly important in the modulation of the trafficking of mGluRs [67] as well as in the regulation of dendritic spine formation [68]. Homer is particularly relevant in the modulation of mGluR-dependent LTD [69,70], can also participate in the stabilization of the post synaptic density (PSD), and influences the endocytosis of NMDARs and AMPARs [66,71].

ESR1 encodes for the estrogen receptor alpha, ER-α. ER-α is present in AD-affected regions like the hippocampus, the basal forebrain, and amygdala [72] and helps in maintaining cholinergic neurotransmission [73,74]. A postmenopausal decrease in estrogen levels is a well-known AD co-risk factor as the hormones favor the brain catalysis of Aβ by regulating the expression of neprilysin, an enzyme that promotes Aβ degradation [75]. Moreover, ER-α polymorphisms are associated with both familial and sporadic AD [76] and decrements of the receptor mRNA splice variants have also been detected with higher frequency in AD female subjects [77]. These changes may help to explain why estrogen-replacement therapy fails to rescue cognitive functions in AD patients [78].

The transcriptional co-activator EP300 plays an important role in regulating gene expression in a number of different cell types [79] and is regulated by Presenilin 1 (PS1), an endoplasmic reticulum/Golgi transmembrane protein whose mutations have been associated with early-onset familial Alzheimer’s disease ([FAD; [80]). P300 promotes a signalling mechanism that is important for long-term memory formation and neuronal survival. The regulation of P300 activity by wild-type PS1 and not by mutant PS1 indicates a partial loss of function in AD that may lead to memory loss and neurodegeneration [79].

**Downregulated genes of the second network.** CDH1 belongs to the cadherin superfamily and encodes for cadherin1, a protein that is involved in neuronal apoptosis. Depletion of cadherin 1 leads to apoptotic neuronal death by favoring the re-entry or reactivation of the cell cycle [81], an important phenomenon as aberrant cell cycle reactivation has been described in AD neurons. Finally, it must be noted that, in cortical cultured neurons, overexpression of Cdh1 results in neuroprotection against Aβ toxicity [82,83].

Actins are key proteins involved in the maintenance of the cytoskeleton as well as in the stabilization of synapses [84]. Actin
filaments can have a particular pathogenic role in AD as they are associated with apolipoprotein E, AβPP, PS1, and the tau protein [46,85]. Actin rods have been shown in autopic samples from AD patients. Moreover, actin is associated with phosphorylation of the synaptic protein, cofilin, and participates in the synaptic structural changes that underlie LTP in vivo and in vitro [86]. In summary, a down expression of actin may affect synaptic efficacy and ultimately impair the molecular machinery that is responsible for learning and memory.

**Downregulated genes of the third network.** PKC can play an important role in AD as its activation promotes the non-amyloidogenic cleavage of AβPP by directly activating the γ-secretase pathway or through an upstream involvement of the MAP kinases ERK1/2 [87]. PKC also inhibits the activity of the β-site AβPP-cleaving enzyme 1, BACE-1, thereby reducing Aβ biosynthesis [88]. PKC dysfunction can also interfere with synaptic functioning. Phosphorylation of key synaptic proteins like adducin, statmin and myristoylated alanine-rich C-kinase substrate (MARCKS) by PKC and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) can severely affect spine integrity. Interestingly, the AD brain appears to suffer from a deficit in PKC activation rather than from a defective PKC expression [89]. Furthermore, a decrease in PKC activation leads to enhanced levels of phosphorylated tau through a reduced PKC-mediated inhibition of GSK-3β [90].

The Shank family of synaptic proteins are particularly important as molecular scaffolds that control the integrity of the postsynaptic density (PSD) and handle the synaptic trafficking of glutamate receptor subunits [91]. A decrease in Shank expression can favor a dysfunctional synaptic rearrangement of NMDA and AMPA receptors and ultimately impair LTP and synaptic efficiency.

**Downregulated genes of the fourth network.** CD3 encodes for the T-cell receptor zeta, a subunit of the T-cell
receptor-CD3 complex which is responsible for recognizing antigens in different intracellular signal-transduction pathways. CD3 positive T-cells induce microglial activation and actively participate in the AD development [92]. However, it must be noted that a significant decrease of CD3(+) lymphocytes can also be responsible for the general decline of immune activity that is observed in AD [78].

In the brain, the glutathione transferase (GST) promotes the cellular response that protect against oxidative injury [93]. Decreased GST activity has been found in the brain of AD patients and has been linked to AD-related neuronal death [94,95]. Furthermore, GST polymorphisms, such as homozygous deletions in GSTM1 and GSTT1 genes, are encoding for defective proteins with less efficient enzymatic activities. These GST polymorphisms have been found with higher frequency in patients affected by late onset AD [96].

Network analysis
Upregulated genes like ARRB1, NOS, c-JUN, and PL2A fit in networks that are linked to neuronal apoptosis. In line with this trend, we also found a parallel downregulation of some neuroprotective and antiapoptotic genes like EP300, CDH1, and GST. Furthermore, our networks analysis indicates that Aβ-Al can promote changes of genes like APLP1, Furin, Clathrin, and IDE that favor alterations of AβPP processing and genes like CaMKII, GRIN1, and GRIN2c that modulate glutamatergic neurotransmission. We also detected changes in c-JUN and PL2A that can influence the inflammatory response as well as an overexpression of TGFβ1, MEF2, and NR41 that can promote a protective compensatory response.

Finally, we find the downregulation of key genes like SHANK 2 and actin that are involved in the maintenance of the structural and functional integrity of synapses.

Pathway analysis
Looking at pathways, we found a IPA-inferred pathway that is related to [Ca²⁺], homeostasis (Fig. 4A). The issue of [Ca²⁺] dyshomeostasis is particularly relevant to AD. Recent findings suggest that Ca²⁺ signaling is altered in AD long before the appearance of macroscopic pathological changes [97,98]. [Ca²⁺] rises might affect formation of AβPP and Aβ [99,100] and promote accumulation of intraneuronal Aβ [97]. Perturbation of [Ca²⁺] levels might also interfere with neurofibrillary tangle formation [101,102]. An altered [Ca²⁺] homeostasis in aging neurons can have important functional consequences and impair synaptic plasticity as well as learning or memory [102]. Some of the genes, like CRAC and CASQ that we found changed by Aβ-Al can also promote [Ca²⁺] deprivation. [Ca²⁺] deprivation can be deleterious too as neurons depleted of Ca²⁺ can undergo death and activate the apoptotic machinery [103,104].

The second pathway inferred by IPA indicates an overall increase of glutamatergic neurotransmission which is interconnected with the issue of [Ca²⁺], dyshomeostasis. Glutamate activates post-synaptic ionotropic receptors and promotes neurotoxic [Ga²⁺], rises. Interestingly, we have previously shown that Aβ-Al elicits the formation of oligomers that selectively increase NMDAR-mediated [Ca²⁺] rises [8]. Excessive [Ca²⁺], rises trigger robust oxidative stress, [101,105] and make neurons more vulnerable to excitotoxicity as free radicals inhibit glutamate reuptake [106]. Aβ-Al can potentiate such loop, not only by increasing NMDAR-evoked currents (as well as the expression of NMDARs and AMPARs; [8]), but also by down regulating glutamate transporters that mediate glutamate reuptake. Thus, glutamate and Aβ-Al can operate synergistically to promote excitotoxic [Ca²⁺], rises and oxidative stress and set the stage for a self-perpetuating harmful loop. It must also be emphasized that oxidative stress can enhance tau phosphorylation and tau-dependent pathology which further exacerbates Aβ-driven pathology [97,107]. In conclusion, the changes in the gene expression profile triggered by Aβ-Al lend support to the idea that this complex is largely involved in the molecular machinery that regulates neuronal as well as synaptic dysfunction and loss (summarized in Figure 6). Aβ-Al seems to modulate the expression of genes that are critical in controlling glutamatergic transmission, Ca²⁺ homeostasis as we have previously shown [108], oxidative stress, inflammation, and neuronal apoptosis. All these processes are key steps in the development of AD pathology. Our analysis can help to unravel the blueprint of the molecular determinants that are set in motion by toxic exposures to the Aβ-Al complex and offer a new perspective on how Al can play a relevant role in AD pathogenesis.

Materials and Methods
Preparation of Aβ-metal complexes
1.0 mg of synthetic Aβ1–42 was dissolved to 1 mM in hexafluorosopropanol (HFIP) for 40 min at room temperature. After this incubation, the Aβ1–42 solution was separated into aliquots in microcentrifuge tubes. Hexafluorosopropanol was removed under vacuum in a Speed Vac (Savant Instruments) and lyophilized peptide film was stored desiccated at –20°C. Immediately prior to use, the HFIP-treated aliquots were completely re-suspended in distilled water to a concentration of 50 μM (following a modified protocol from Dahlgren et al., 2002). The Aβ-Al complex was prepared with a 24 h dialysis against a metal solution containing Al(CH3H5O3)3 at T = 4°C using Spectra/Por5 Float-A-Lyzer R tubes (Spectrum Labs) with 1000 Molecular Weight Cut Offs (MWCO). The Aβ-Al complex was then dialysed against water (three water changes) for 24 h in order to remove the excess of metal not bound to the peptide. The same treatment was also performed with Aβ alone. SH-SY5Y cells were treated with Aβ or Aβ-Al complex at 0.5 μM peptide concentration for 24 h. Aliquots of Aβ and Aβ-Al complex were taken, after dialysis, at 48 h incubation time to be analyzed by electron microscopy. Metal detection was done by atomic absorption (electrothermal atomic absorption spectrometry or flame atomic absorption spectrometry) and size exclusion chromatography. After 48 h dialysis at 4°C, many short and irregular protofibrillar structures were present in the Aβ sample as the consequence of self aggregation while few fibrils were observed. By contrast, the Aβ-Al complex was characterized by a large population of small oligomeric aggregates that as previously shown [8] possess marked neurotoxic effects. Thus, as previously described [8], Al freezes the oligomeric state of Aβ.

Cell cultures
SH-SY5Y human neuroblastoma cells were purchased from ECACC (European Collection of Cell Culture, Salisbury, UK). SH-SY5Y human neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (MEM), F-12 (1:1) with L-Glutamine and 13 mM Hepes (Gibco, Carlsbad, CA USA) at 37°C with 5% CO2 in a humidified atmosphere (90% humidity). The medium was replaced every 2 days. Penicillin (100 units/ml), Gibco, Carlsbad, CA USA) and streptomycin (100 μg/ml; Gibco, Carlsbad, CA USA), 15% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO), and MEM non-essential amino acids (100x; Sigma Aldrich, St. Louis, MO) were added to the medium. 0.25% Trypsin-EDTA solution and phosphate buffered saline (PBS) were
obtained from Sigma Aldrich (St. Louis, MO). SHSYSY cells were plated onto 6-well plates. The day after this plating, the culture medium was replaced with the same medium with 2% FBS containing Aβ1–42 or Aβ1–42-Al at 0.5 mM peptide concentration. The cells were incubated with different Aβ or Aβ-Al for 24 h. The cell treatment was also performed in the presence of metal (Al) at a concentration 10-fold higher than the peptide concentration.

RNA isolation, quality control and labeling

Total RNA of neuroblastoma cells untreated or treated with various Aβ and Aβ-Al was isolated using the Qiagen RNA/DNA Mini Kit (Qiagen S.p.A, Milan, Italy) following the manufacturer’s instructions. 1 ml total RNA aliquots were used for quality control by capillary electrophoresis using the RNA 6000 Nano LabChip and the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). All RNA samples used in this study showed no sign of degradation. 1 μg of total RNA was amplified following the Superscript™ Indirect RNA Amplification System Kit (Invitrogen™): total RNA was reverse transcribed into single-stranded complementary DNA (cDNA) using oligo(dT) primer containing a T7 promoter and Superscript III reverse transcriptase. RNase H was then added together with E. coli DNA polymerase I and E. coli DNA ligase, followed by a short incubation in order to achieve synthesis of the second-strand cDNA. The purified double-strand cDNA served as the template for the in vitro transcription reaction, which was carried out overnight in the presence of T7-RNA polymerase. This step generated antisense RNA (aRNA) molecules complementary to the original mRNA targets, incorporating amino-allyl UTP (aa-UTP) into the aRNA. 10 μg of purified aRNA was coupled with Alexa Fluor 555 or Alexa Fluor 647 dyes (Invitrogen, Grand Island, NY) according to the manufacturer instructions.

Microarray

Labeled aRNA was dissolved in 90 μl of hybridization buffer, denaturated at 90°C for 3 min and applied directly to human OpArrays DNA microarray slides containing 35130 spotted oligonucleotide sequences representing 29166 different human genes (Operon Biotechnologies, Inc.). Each slide was hybridized with treated (either Aβ, Aβ-Al, or Al) and untreated (sham) samples. Microarray hybridization was carried out in an ArrayBooster Hybridization Station (Advalytix, Brunnthal, Germany). The reaction was carried out overnight at 42°C. Posthybridization washing was performed according to the microarray slides manufacturer’s instructions. Two replicates of each experiment were performed using different microarray slides.

Figure 6. Summary of genes, biological functions, and pathways associated with gene expression changes triggered by the exposure to the Aβ-Al complex in SH-SY5Y cells.

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in which sample and control RNA, labeled either with Alexa Fluor 555 or Alexa Fluor 647 fluorochromes, were crossed in both combinations (dye-swapping procedure). Each experiment used material from the same cell culture and therefore represents a technical replicate. The microarrays slides were scanned using a GenePix 4000B laser scanner ( Molecular Devices Corp., Sunnyvale, CA, USA) and images were processed using Genepix 6.0 software.

Microarray data are deposited in the GEO public database (accession number:GSE23000). All data are MIAME compliant.

**Statistical analysis**

Statistical analysis of the microarray data was performed using Acuity 4.0 software ( Molecular Devices Corp., Sunnyvale, CA, USA). Initial results were normalized using Lowess algorithm and filtered to exclude extreme outliers and flags. We also excluded any spot that had no detectable response on all slides. The log-ratios of expression were calculated as the base 2 logarithm of the ratios of background-corrected intensity medians of red dye over green dye intensities. In order to obtain a single expression value for each gene, the internal replicates (1 to 14) were plotted versus the technical replicates (6 for Aβ treatment and 2 for AI and Aβ-Al complex treatment). The X-Y scatter plot confirmed that the data were generally reproducible but we observed the presence of extreme outlier values. Then we excluded the outliers and the values of spots replicates within arrays were averaged using a trimmed mean. We excluded any spot that had no detectable response in all three treatments. Consequently, the final dataset was composed of 28676 genes. In order to identify important genes with great statistical confidence, statistical testing and fold change criteria were employed simultaneously. A gene was considered to be differentially expressed if it had an absolute value of a log-ratio higher or equal to 0.5, representing a fold-change of 1.4 in transcript quantity. Student’s t-test was applied and statistical significance set at p<0.05.

**TaqMan real-time quantitative PCR (qRT-PCR)**

A qRT-PCR analysis was performed on SH-SY5Y neuroblastoma treated with Aβ, Aβ-Al complex or Al alone to verify the gene expression profile of APLP1, APLP2, MAPT and AβPP, the genes we identified by microarray analysis. Real time quantitative PCR was carried out in a total volume of 25 μl containing 1X TaqMan Universal PCR Master mix, no AmpErase UNG and 2.5 μl of cDNA using the TaqMan assay (TAB) on ABI 7300 Sequence Detection System (ABI, Foster City, CA). Gene-specific primers and probe sets for each gene (APLP1; Hs00194306-M1) (APLP2; Hs00153778-M1) (AβPP; Hs00069098-M1) (MAPT; Hs00214391-M1) (GAPDH; Hs99999905-M1) were obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). Duplicate samples were run for each gene along with a no-template control. The housekeeping gene GAPDH was used as an internal control to normalize the expression of target genes. The real time amplifications included 10 minutes at 95°C (AmpliTaq Gold activation), followed by 40 temperature cycles for 15 seconds at 95°C and for 1 minute at 60°C. Relative expression levels were calculated for each sample after normalization against the housekeeping gene GAPDH, using the ΔΔCt method for comparing relative fold expression differences [109].

**Supporting Information**

**Figure S1** Graph bars show mRNA levels of APLP1 (a) and APLP2 (b) as measured by real-time PCR in SH-SY5Y cell exposed to the Aβ-Al complex, Aβ, or Al (*indicates p<0.0001 vs control; # indicates p<0.0001 vs Aβ and Al, n = 3). Values are expressed as means ±S.E.M. (TIF)

**Table S1** List of genes (1535) selectively overexpressed upon exposure to Aβ-Al compared to exposures to Aβ or Al alone. (DOC)

**Table S2** List of genes (1815) selectively downexpressed upon exposure to Aβ-Al compared to exposures to Aβ or Al alone. (DOC)

**Table S3** List of the overexpressed genes found in the first network (see Fig. 2A). (DOC)

**Table S4** List of the overexpressed genes found in the second network (see Fig. 2B). (DOC)

**Table S5** List of the overexpressed genes found in the third network (see Fig. 2C). (DOC)

**Table S6** List of the overexpressed genes found in the fourth network (see Fig. 2D). (DOC)

**Table S7** List of the downexpressed genes found in the first network (see Fig. 3A). (DOC)

**Table S8** List of the downexpressed genes found in the second network (see Fig. 3B). (DOC)

**Table S9** List of the downexpressed genes found in the third network (see Fig. 3C). (DOC)

**Table S10** List of the downexpressed genes found in the fourth network (see Fig. 3D). (DOC)

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**Author Contributions**

Conceived and designed the experiments: PZ DD. Performed the experiments: DD KF MTV LDC. Analyzed the data: VG KF SLS. Contributed reagents/materials/analysis tools: VG DD KF MTV LDC SLS PZ. Wrote the paper: VG SLS.
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