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Aging versus Alzheimer’s Disease-Like Pathology

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Abstract

Aging is the primary risk factor of neurodegenerative disorders such as Alzheimer’s disease (AD). However, the molecular events occurring during brain aging are extremely complex and still largely unknown. For a better understanding of these age-associated modifications, animal models as close as possible to humans are needed. We thus analyzed the transcriptome of the temporal cortex of the primate Microcebus murinus using human oligonucleotide microarrays (Affymetrix). Gene expression profiles were assessed in the temporal cortex of 6 young adults, 10 healthy old animals and 2 old, “AD-like” animals that presented β-amyloid plaques and cortical atrophy, which are pathognomonic signs of AD in humans. Gene expression data of the 14,911 genes that were detected in at least 3 samples were analyzed. By SAM (significance analysis of microarrays), we identified 47 genes that discriminated young from healthy old and “AD-like” animals. These findings were confirmed by principal component analysis (PCA). ANOVA of the expression data from the three groups identified 695 genes (including the 47 genes previously identified by SAM and PCA) with significant changes of expression in old and “AD-like” in comparison to young animals. About one third of these genes showed similar changes of expression in healthy aging and in “AD-like” animals, whereas more than two thirds showed opposite changes in these two groups in comparison to young animals. Hierarchical clustering analysis of the 695 markers indicated that each group had distinct expression profiles which characterized each group, especially the “AD-like” group. Functional categorization showed that most of the genes that were up-regulated in healthy old animals and down-regulated in “AD-like” animals belonged to metabolic pathways, particularly protein synthesis. These data suggest the existence of compensatory mechanisms during physiological brain aging that disappear in “AD-like” animals. These results open the way to new exploration of physiological and “AD-like” aging in primates.

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Introduction

In EU, life expectancy is about 83 years for women and 76 for men, and it is expected to further increase. This trend will have major economic and social impacts due to the prevalence of age-related diseases. “Normal” age-related cognitive changes have been traditionally ascribed to cell loss in the brain. However, experimental evidence is now accumulating suggesting that synaptic rearrangement, rather than cell loss, causes critical age-related brain changes [1–3]. Most elderly individuals experience no major, or very limited functional impairment thus highlighting the human brain ability to compensate for potential cognitive decline. In other words, although aging may be a cause of cognitive alteration and is the main risk factor for Alzheimer’s disease (AD) [4], they are clearly two separated processes. AD is not accelerated aging and therefore it is important to be able to distinguish physiological from pathological aging. The discovery of genes, which are differentially expressed in normally aging and AD brains, could provide a tool to identify biomarkers [5] that can differentiate between physiological and pathological brain aging and which might help to identify new therapeutic targets.

Non-human primates constitute a valuable tool for studies on brain aging because of their lifespan and their closeness to humans. We propose to take advantage of the grey mouse lemur, Microcebus murinus, which is a pro-simian primate whose lifespan in captivity can reach 12–13 years. In grey mouse lemurs, the 50% survival point occurs at 60 months (i.e., at the age of 5 years) and allows defining the adult and elderly part of the population [6]. Animals older than 5 years of age show desynchronized biological rhythms, motor activity and sleep-wake cycles [6], [7]. Importantly, during aging, some lemurs develop, like humans, pathognomonic signs of AD such as presence of β-amyloid plaques [8] with a prevalence of 5% (data unpublished), tau protein aggregation...
and cerebral atrophy [10]. Animals with cerebral atrophy present the most severe neuropathology associated with a progressive and consistent pattern of neuronal-glial alterations [10]. Moreover, this animal model can be used to investigate cognitive deficits [11–14].

We thus analyzed the gene expression profiles in the temporal cortex of young adult, healthy elderly and old lemurs with ß-amyloid plaques ("AD-like" group) in order to identify genes involved in physiological and pathological aging. Since Microcebus murinus microarrays do not exist, we decided to use Affymetrix human genome chips since recent studies have illustrated the feasibility of detecting non-human primate brain transcripts using human genome chips [15–18]. We chose the temporal cortex because this region is connected to the hippocampus and to the frontal cortex, which are critical structures for learning and memory in AD. Analysis of the microarray data indicates that the temporal cortex has different gene expression profiles in young, old and "AD-like" animals and that several genes are differentially expressed in healthy aged and "AD-like" animals. Specifically, genes involved in metabolism, particularly in protein synthesis, were up-regulated during physiological brain aging, whereas in "AD-like" brains genes involved in protein synthesis and nuclear activity were often down-regulated.

Results

Histological characterization of young adult, elderly and "AD-like" brain in Microcebus murinus

Before investigating the possible differences of gene expression in the temporal cortex of young adult, elderly and "AD-like" grey mouse lemurs, we compared the anatomical shape of the brains from the three groups (Fig. 1). We did not observe any difference

Table 1. Genes detected in the temporal cortex of Microcebus murinus.

| Age (yr) | Males | Females | Number of detected genes |
|---------|-------|---------|--------------------------|
| Young 1 | 1     | 2       | 4                        |
| 8914.67 | 1482.68 |
| Old 7.8 | 3     | 7       | 9456.20                   |
| 1378.93 |
| AD-like 10 | 0  | 2       | 8259.00                   |
| 45.25   |
| All 5   | 13    |         | 9142.67                   |
| 1349.59 |

Transcripts detected in temporal cortex samples from young adult, old and "AD-like" animals by hybridization to the Affymetrix HG-U133 Plus 2.00 Array. The ages and the detected genes were expressed as mean ± SD.

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in the thickness of the cerebral cortex and in the shape of the lateral ventricles between young (Fig. 1A) and healthy old animals (Fig. 1B). Conversely, in “AD-like” animals, we detected cortical atrophy, lateral ventricle dilatation (Fig. 1C) and β-amyloid plaques (Fig. 1D, 1E, 1F and 1G).

### Detection of transcripts with Affymetrix human genome chips

In order to assess the feasibility of using human genome arrays to study gene expression in the temporal cortex of *Microcebus murinus*, we first sequenced about 150 genes of the grey mouse lemur and compared them with their human homologues. They all presented a percentage of identity with their human counterparts that varied from 88% to 96% (data not shown). These results supported our choice of using the Affymetrix HG-U133 Plus 2.0 human microarray to investigate the pattern of gene expression in the temporal cortex of young adult, old and “AD-like” grey mouse lemurs (Table 1). The overall rate of genes with a “present” detection call (P) for each group was about 20%, with a slightly higher number of genes (9,456 ± 1,378) detected in healthy aged animals, and a slightly lower number of genes (8,259 ± 45) expressed in the “AD-like” group in comparison to the young adult group (8,914 ± 1,482). Moreover, we noticed a lower SD in

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**Figure 2. Schematic representation of the different analyses.** Microarray data were filtered to detect the present transcripts (P). Then the filtered data were processed by SAM. ANOVA was performed with the (P) data and with the data sorted by SAM and the 47 P selected genes were analyzed by PCA. Finally, the 47 and the 695 genes were investigated by clustering and were classified by functional categories.

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both “AD-like” animals, suggesting a better homogeneity of gene expression within this group. In human somatic tissues, which also included brain samples, the mean rate of genes with P call was 21278 (range: 7142–28389). Hence, although the percentage of genes detected by the HG-U133 Plus 2.0 microarray was lower in grey mouse lemur samples, it was still within the range of values observed in human samples.

Analysis of age-dependent gene expression changes in the temporal cortex

In order to characterize the expression profiles of adult, old and “AD-like” temporal cortex of Microcebus murinus, we first filtered the raw data (see Figure 2 for a schematic description for the analytical approach) and kept only the transcripts (n = 14,911) which had a “P” call in at least 3 samples. We then used the Significance Analysis of Microarrays (SAM) method to identify genes that were differentially expressed when comparing the three groups together or when assessing only healthy elderly and young or “AD-like” animals. The comparison of the three groups identified 1,055 transcripts with a false discovery rate (FDR) up to 45%. Since this risk of false positive was high, we assessed the significance of the changes in gene expression by ANOVA. The ANOVA analysis with a cut off at p = 0.05 identified 695 genes with a significant gene expression change (i.e. 4.7% of expressed genes) and this number was reduced to 152 when the cut off was set at p = 0.01 (i.e. 1% of the expressed genes) (Fig. 2 and Table S1).

Table 2. Number of genes identified by SAM as differentially regulated in the temporal cortex of old animals.

| Delta | Significant genes | FDR% |
|-------|-------------------|------|
| 0.76  | 6                 | 0.00 |
| 0.74  | 14                | 6.84 |
| 0.64  | 44                | 10.88|
| 0.56  | 53                | 12.65|
| 0.56  | 70                | 13.68|
| 0.56  | 3                  | 17.31|
| 0.56  | 105               | 20.06|
| 0.56  | 116               | 20.64|
| 0.56  | 48                | 21.35|
| 0.56  | 177               | 22.18|
| 0.56  | 213               | 23.83|
| 0.56  | 233               | 24.25|
| 0.56  | 334               | 27.24|
| 0.56  | 399               | 31.08|
| 0.56  | 321               | 34.74|
| 0.56  | 605               | 37.67|
| 0.56  | 710               | 41.41|

Delta table: number of genes with significant expression change in brain of aged lemurs detected by SAM analysis of expression data from temporal cortex samples of 10 healthy old lemurs and 6 young adults. The delta parameter is the threshold used to select genes that are significantly differentially expressed. The false discovery rate (FDR, expressed in %) is defined by the number of false positive genes divided by the number of genes identified as differentially expressed. Higher delta values lower the FDR but also reduce the number of selected genes. Delta table. doi:10.1371/journal.pone.0012770.t002

Table 3. Genes identified by SAM as differentially regulated in the temporal cortex of old animals.

| Probesets | Name       | FC  | p-value | Function |
|-----------|------------|-----|---------|----------|
| 205061_s_at | EXOSC9    | 1.77 | ****    | Prot. Synthesis |
| 0200936_at  | RPL8      | 1.58 | ***     | Prot. Synthesis |
| 200817_x_at | RPS10     | 1.56 | ***     | Prot. Synthesis |
| 226131_s_at | RPS16     | 1.59 | ***     | Prot. Synthesis |
| 211487_x_at | RPS17     | 1.70 | ***     | Prot. Synthesis |
| 212270_x_at | RPL17     | 1.54 | ***     | Prot. Synthesis |
| 214003_x_at | RPS20     | 1.65 | ****    | Prot. Synthesis |
| 200834_s_at | RPS21     | 1.65 | ***     | Prot. Synthesis |
| 218830_at  | RPL26L1   | 1.59 | **      | Prot. Synthesis |
| 219762_s_at | RPL36     | 1.85 | ***     | Prot. Synthesis |
| 212863_x_at | CTBP1     | 1.47 | **      | Prot. Synthesis |
| 200757_s_at | CALU      | 1.37 | ***     | Prot. Maturaton |
| 222578_s_at | UBE1DC1   | 1.58 | ***     | Proteolysis |
| 201322_at  | ATP5B     | 1.38 | **      | Mitoch. Metabolism |
| 207507_s_at | ATP5G3    | 1.56 | *       | Mitoch. Metabolism |
| 200818_at  | ATP5O     | 1.49 | **      | Mitoch. Metabolism |
| 201226_at  | NDUFB8    | 1.63 | **      | Mitoch. Metabolism |
| 201227_s_at | NDUFB8    | 1.65 | ***     | Mitoch. Metabolism |
| 35201_at   | HNRPL     | 1.34 | *       | RNA related |
| 218117_at  | RBX1      | 1.59 | ***     | Nuclear factor |
| 226465_s_at | SON       | 1.72 | ***     | Nuclear factor |
| 204009_s_at | KRAS      | 1.38 | ***     | Epigenetic |
| 207721_s_at | HINT1     | 1.58 | **      | Epigenetic |
| 1555961_a_at | HINT1    | 1.68 | ***     | Epigenetic |
| 224415_s_at | HINT2     | 1.47 | ***     | Epigenetic |
| 222540_s_at | HBXAP     | 1.38 | ***     | Epigenetic |
| 204745_s_at | MT1G      | 2.04 | ***     | Cell cycle regulation |
| 206461_s_at | MT1H      | 2.02 | ***     | Cell cycle regulation |
| 218206_x_at | SCAND1    | 1.45 | ****    | Cell proliferation |
| 208864_s_at | TXN       | 1.56 | ***     | Cell proliferation |
| 205352_at  | SERPIN1   | 1.50 | ***     | Neurogenesis |
| 232520_s_at | NSFL1C    | 1.48 | ***     | Synapse |
| 202670_at  | MAP2K1    | 1.59 | ****    | Kinase |
| 226888_at  | CSNK1G1   | 1.35 | ****    | Kinase |
| 217848_s_at | PP        | 1.69 | ****    | Phosphatase |
| 205809_s_at | WASL      | 1.80 | **      | Transduction |
| 219392_x_at | FLJ11029  | 1.36 | ***     | Unknown |
| 229302_at  | MGC33926  | 1.46 | ****    | Unknown |
| 230076_at  | FLJ10156  | 1.47 | ****    | Unknown |
| 225956_at  | LOC153222 | 1.54 | *       | Unknown |
| 218859_s_at | C20orf6   | 1.88 | **      | Unknown |
| 241933_x_at | —         | 1.90 | ****    | Unknown |
| 218011_at  | —         | 1.57 | ****    | Unknown |

List of 44 transcripts detected with a delta value set at 0.64 and a FDR of about 10%. Genes were classified according to their function: metabolism, nuclear activity, neurotransmission–synaptic plasticity, signaling pathways and unknown functions. P-value of the Student’s t-test: 0.05 (*); 0.01 (**); 0.005 (***) ; 0.001 (****); 0.0005 (*****); FC: fold change. The 6 genes detected with 0% FDR were noted in bold. doi:10.1371/journal.pone.0012770.t003
On the other hand, comparison of the gene expression data from the young and the old group by SAM sorted 44 transcripts (FDR about 10%, Table 2), which were significantly up-regulated (fold change between 1.37 and 3.82) in healthy aging animals (Table 3). These genes are mainly involved in protein synthesis, mitochondrial metabolism, and nuclear regulation. Conversely, comparison of the gene expression data from “AD-like” and healthy old animals indicated that 40 genes were strongly down-regulated (fold change between 2.0 and 3.3) in the “AD-like” group (Tables 4 and 5), with the exception of GK001 that was up-regulated. Among these genes, only a fraction plays a role in protein synthesis and nuclear regulation.

When for the comparison of the three groups a more stringent delta cutoff was chosen (from 0.058 to 0.03) the number of genes identified as differentially expressed in the three groups was reduced to 47 and among them were included the 28 genes that had been already sorted by the two SAM analyses using the data from young and healthy old animals or healthy old and both “AD-like” animals (Tables 6 and 7).

Principal component analysis of the 47 genes, which were identified as differentially expressed in the three groups by SAM, confirmed that they could clearly separate young, healthy old, and “AD-like” animals (Fig. 3). The first component represented the gene expression signature with a score of 64.59% of the variance in the three groups. Healthy aged animals could be differentiated from young ones with a score of 43.85% (Fig. 3A, axis 1), and in the three groups. Healthy aged animals could be differentiated from young and healthy old animals or healthy old and both “AD-like” animals (Fig. 3B). Genes located to the left of axis 1 characterized the group of healthy aged animals. Most of them are involved in protein synthesis (RPS16, 17, 19, 20, 21, RPL26L1 and 36), mitochondrial metabolism (NADH dehydrogenase complex, NDUFB8) or in the regulation of transcription (SCAND1, HNRPL, HINT1-2, KRAS).

### Table 4. Number of genes identified by SAM as differentially regulated in “AD-like” temporal cortex samples.

| Delta | Significant genes | FDR% |
|-------|-------------------|------|
| 0.46  | 2                 | 0.00 |
| 0.38  | 27                | 17.42|
| 0.36  | 37                | 19.06|
| 0.35  | 40                | 21.16|
| 0.28  | 52                | 23.51|
| 0.24  | 84                | 30.79|
| 0.24  | 94                | 29.51|
| 0.23  | 110               | 30.78|
| 0.21  | 172               | 35.27|
| 0.20  | 208               | 35.72|
| 0.20  | 244               | 36.61|
| 0.19  | 341               | 37.78|
| 0.19  | 460               | 40.07|
| 0.17  | 569               | 42.15|
| 0.17  | 765               | 43.89|
| 0.16  | 836               | 45.28|
| 0.15  | 968               | 48.92|
| 0.14  | 1073              | 51.27|

Delta table: number of genes with significant expression changes in aged animals detected by SAM analysis of expression data from temporal cortex samples from 2 “AD-like” lemurs and 10 healthy old animals. 
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### Table 5. Genes identified by SAM as differentially regulated in “AD-like” temporal cortex samples.

| Probesets | Name            | FC   | p-value | Function                  |
|-----------|-----------------|------|---------|---------------------------|
| 211487_x_at | RPS17          | −2.07 *** | Prot. synthesis          |
| 200834_s_at | RPS21          | −1.60 **** | Prot. synthesis          |
| 225672_at | GOLGA2         | −1.88 *   | Prot. maturation         |
| 38710_at  | OTUB1          | −1.51 *   | Proteolysis              |
| 35201_at | HNRPL          | −1.61 ***** | RNA related              |
| 203752_s_at | JUNO          | −1.86 *   | Transcription            |
| 227772_at | LAT51          | −1.98 *   | Transcription            |
| 200055_at | TAF10          | −1.90 *** | RNA related              |
| 209431_s_at | ZNF278        | −2.53 ***** | Nuclear factor          |
| 218977_s_at | SECP43        | −2.71 ***** | Nuclear factor          |
| 48580_at  | CXXC1          | −2.84 *   | Nuclear factor           |
| 223132_s_at | TRIM8         | −1.74 *   | Nuclear factor           |
| 208676_s_at | PA2G4         | −1.90 *   | Cell cycle regulation    |
| 218034_at | TTC11          | −1.65 **  | Apoptosis                |
| 219888_at | SPAG4          | −3.34 **  | Cytoskeleton             |
| 203069_at | SV2A           | −1.64 **  | Synapses                 |
| 225058_at | GPR108         | −2.23 **  | Synapses                 |
| 209982_s_at | NRXN2         | −2.59 *** | Synapses                 |
| 229309_at | ADRB1          | −1.79 *   | Synapses                 |
| 206397_x_at | GDF1          | −2.01 *   | Growth factor            |
| 210185_at | CACNB1         | −1.70 *   | Ion channel              |
| 222432_s_at | GK001         | 2.65 ***** | Signal. Transd.          |
| 213108_at | CAMK2A         | −1.65 ***** | Kinase                   |
| 215903_s_at | MAST2         | −2.22 ***** | Kinase                   |
| 228302_x_at | CaMKIINalpha  | −3.09 *** | Kinase                   |
| 209945_s_at | GSK3B         | −1.97 *   | Kinase                   |
| 200822_x_at | TP1           | −1.87 *** | Phosphate metabol.       |
| 33197_3_at | GAPD           | −1.33 *   | Phosphate metabol.       |
| 225519_at | PPP4R2        | −2.08 ***** | Phosphatase              |
| 227325_at | LOC255783      | −5.11 ***** | Unknown                  |
| 228935_at | LOC283400      | −5.20 ***** | Unknown                  |
| 210408_s_at | CPN6         | −2.06 ***** | Unknown                  |
| 217033_x_at | —             | −1.68 ***** | Unknown                  |
| 1554429_a_at | DMWD         | −1.87 ****  | Unknown                  |
| 219392_x_at | FLJ11029     | −1.79 ****  | Unknown                  |
| 48106_at  | —              | −2.01 ***  | Unknown                  |
| 219910_at | HYPE           | −1.57 *   | Unknown                  |
| 207435_s_at | SRRM2        | −1.97 *   | Unknown                  |
| 218089_at | C20orf4       | −1.92 *   | Unknown                  |
| 218429_s_at | FLJ11286     | −1.72 *   | Unknown                  |

List of the 40 transcripts sorted using a delta set at 0.35 and a FDR of 21%. All were down-regulated in AD, but for GK001. Genes were classified according to their function: metabolism, nuclear activity, neurotransmission–synaptic plasticity, signaling pathways and unknown functions. FC, fold change; P value: 0.05 (*); 0.01 (**); 0.05 (**); 0.001 (****); 0.0005 (*****). The 2 genes detected with 0% FDR were noted in bold. doi:10.1371/journal.pone.0012770.t005
axis 2 characterized the “AD-like” group. Some of these genes play a role in neurotransmission (STXBPI), intracellular trafficking (SNAGI), regulation of transcription (ZBTB11) or in epigenetic processes (HAT1). However, also genes, which are not yet reported as having brain-related functions, such as SPAI, which is expressed in mature lymphocyte B cells, contributed to delineate the “AD-like” group. Calumenin (CALU), a protein which is involved in the regulation of vitamin K-dependent carboxylation of multiple amino-terminal glutamate residues and normally is present at very low levels in the brain, also was up-regulated in the “AD-like” animals. Hierarchical clustering identified clusters of genes according to their cellular functions and distribute them in 4 ontology categories, we could classify the differentially expressed transcripts with significant changes was higher: 1,079 instead of 695 because of less variance due to a better homogeneity of the populations.

The 47 genes sorted by SAM were included in the 695 genes according to their cellular functions and distribute them in 4 ontology categories, we could classify the differentially expressed transcripts with significant changes was higher: 1,079 instead of 695 because of less variance due to a better homogeneity of the populations. Genes with significant expression changes identified when data from the cortex samples of young (Y), healthy aged (A) and “AD-like” (AD) were compared. FC: Fold Change. These genes have been already sorted as regulated by age (see Table 3); B: genes already identified as regulated by AD (Table 5); C: genes regulated by age and by AD (Table 3 and 5).

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Table 6. SAM analysis of the expression data from the three groups.

| Probesets | Name   | FC A/Y | p-value | FC AD/Y | p-value | FC AD/A | p-value | Functions               |
|-----------|--------|--------|---------|---------|---------|---------|---------|-------------------------|
| A         | RPS16  | 1.59   | ***     | 1.19    | NS      | −1.34   | *       | prot synthesis          |
| 214003_s_at | RPS20 | 1.65   | ****    | 1.03    | NS      | −1.60   | ***     | prot synthesis          |
| 218830_at | RPL26L1 | 1.59   | ***     | 1.26    | *       | −1.26   | *       | prot synthesis          |
| 219762_s_at | RPL36 | 1.85   | ****    | −1.02   | NS      | −1.88   | *       | prot synthesis          |
| 200757_s_at | CALU  | 1.37   | *****   | −1.07   | *       | −1.47   | *****   | prot maturation          |
| 201227_s_at | NDUF88 | 1.65   | ****    | 1.20    | **      | −1.37   | ****    | mitoch metab.            |
| 218206_s_at | SCAND1 | 1.45   | *****   | −1.04   | NS      | −1.51   | ***     | cell prol.               |
| 207721_s_at | HINT1 | 1.58   | ***     | 1.13    | NS      | −1.40   | NS      | Epigenetic               |
| 224415_s_at | HINT2 | 1.47   | ***     | −1.04   | NS      | −1.53   | *****   | Epigenetic               |
| 222540_s_at | HBXAP | 1.38   | ***     | 1.09    | NS      | −1.27   | **       | Epigenetic               |
| 204009_s_at | KRAS  | 1.38   | ***     | 1.04    | NS      | −1.32   | *        | Epigenetic               |
| 202670_at | MAP2K1 | 1.59   | *****   | 1.21    | NS      | −1.32   | NS      | Kinase                   |
| 217848_s_at | PP    | 1.69   | *****   | 1.34    | ***     | −1.26   | *        | Phosphatase              |
| 230076_at | FLJ10156 | 1.47  | *****   | 1.10    | NS      | −1.34   | ***     | Unknown                  |
| 241993_x_at | SCAND1 | 1.90   | *****   | 1.35    | NS      | −1.41   | NS      | Unknown                  |
| 218011_at | —      | 1.57   | *****   | 1.09    | NS      | −1.45   | NS      | Unknown                  |
| B         | TRIM8  | −1.06  | NS      | −1.85   | *       | −1.74   | *       | nuclear fact.            |
| 222432_s_at | GKO01 | −1.19  | NS      | 2.23    | **       | 2.65    | *****   | signal transd.           |
| 213108_at | CAMK2A | 1.42   | *       | −1.16   | ****    | −1.65   | *****   | Kinase                   |
| 209945_s_at | GSK3B | 1.43   | *       | −1.38   | NS      | −1.97   | *        | Kinase                   |
| C         | RPS17  | 1.70   | ****    | −1.22   | NS      | −2.07   | ***     | prot synthesis          |
| 200834_s_at | RPS21 | 1.65   | ****    | −1.03   | NS      | −1.60   | ****    | prot synthesis          |
| 35201_at | HNRPL  | 1.34   | *       | −1.20   | *       | −1.61   | ****    | RNA related              |
| 219392_x_at | FLJ1029 | 1.36  | ***     | −1.32   | NS      | −1.79   | ****    | Unknown                  |

Genes with significant expression changes identified when data from the cortex samples of young (Y), healthy aged (A) and “AD-like” (AD) were compared. FC: Fold Change. These genes have been already sorted as regulated by age (see Table 3); B: genes already identified as regulated by AD (Table 5); C: genes regulated by age and by AD (Table 3 and 5).

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Functional categorization and sexually dimorphic changes in aged Microcebus murinus males and females

By assessing the relative changes in expression within gene ontology categories, we could classify the differentially expressed genes according to their cellular functions and distribute them in 4 main modules: 1- Brain plasticity; 2- Signaling pathways; 3- Metabolism and protein synthesis; 4- Nuclear activity (Fig. 6 and Table S2_gene functional categorization). Most of the genes that
Table 7. SAM analysis of the expression data from the three groups.

| Probesets | Name       | FC A/Y | p-value | FC AD/Y | p-value | FC AD/A | p-value | Functions                  |
|-----------|------------|--------|---------|---------|---------|---------|---------|----------------------------|
| 200029_at | RPL19      | 1.69   | ***     | 1.07    | NS      | −1.58   | NS      | prot synthesis             |
| 222091_s_at | UBQLN1     | −1.27  | NS      | 1.61    | **      | 2.04    | ***     | Proteolysis                |
| 204528_s_at | NAP1L1     | 1.09   | NS      | 2.15    | *****   | 2.34    | *****   | RNA related                |
| 213470_s_at | HNRPH1     | −1.52  | ***     | −1.45   | *       | 1.05    | NS      | RNA related                |
| 204847_at | ZBTB11     | 1.34   | **      | 2.17    | *****   | 1.62    | *****   | nuclear fact.              |
| 203138_at | HAT1       | 1.17   | NS      | 2.14    | **      | 1.83    | **      | Epigenetic                 |
| 203845_at | PCAF       | 1.13   | NS      | 1.93    | NS      | 1.71    | NS      | Epigenetic                 |
| 202260_s_at | STXB1P1    | 1.08   | NS      | 1.50    | *****   | 1.40    | *****   | Synapse                    |
| 214441_at | STX6       | −1.67  | *****   | −1.50   | **      | 1.12    | NS      | Synapse                    |
| 232520_s_at | NSFL1C     | 1.48   | ****    | 1.20    | **      | −1.23   | *       | Synapse                    |
| 232663_at | SNAG1      | 1.01   | NS      | 1.51    | *****   | 1.50    | *****   | Synapse                    |
| 205352_at | SERPIN1    | 1.50   | ***     | 1.64    | *       | 1.09    | NS      | Neurogenes.                |
| 222960_at | CACNA1H    | −2.04  | *****   | −1.81   | *       | 1.13    | NS      | Ion channel                |
| 223208_at | KCTD10     | −1.86  | ***     | 1.52    | *****   | 2.83    | *****   | Ion channel                |
| 211302_s_at | PDE4B      | −2.29  | ***     | −4.68   | ***     | −2.05   | NS      | Phosphatase                |
| 1563674_at | SPAP1      | 1.68   | NS      | 4.78    | ***     | 2.84    | **      | Phosphatase                |
| 218021_at | DHR54      | −1.49  | *****   | 1.10    | NS      | 1.64    | **      | Dehydrogen                 |
| 216341_1_at | S100A8     | 1.50   | ***     | 1.64    | *       | 1.09    | NS      | Neurogenes.                |
| 65635_at  | FLJ21865   | −1.30  | ***     | −1.43   | ***     | −1.10   | NS      | Unknown                    |
| 215672_s_at | KIAA0828   | −1.72  | *****   | −1.66   | NS      | 1.04    | NS      | Unknown                    |
| 48106_at  | —          | −1.18  | NS      | −2.37   | *****   | −2.01   | ***     | Unknown                    |
| 240835_at | —          | 1.16   | NS      | 2.26    | *****   | 1.95    | *****   | Unknown                    |
| 1553705_s_at | —         | −1.50  | ***     | −1.07   | NS      | 1.41    | ***     | Unknown                    |

Genes with significant expression changes identified when data from the cortex samples of young (Y), healthy aged (A) and “AD-like” (AD) were compared. FC: Fold Change. These genes have been identified by SAM using the data from the three groups.

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Discussion

Microcebus murinus is a useful primate model of cerebral aging and AD. We already showed that, not only they display pathognomonic lesions for AD and as observed in humans, both lemur with AD-like pathology presented cortical atrophy. Recent studies have shown that brain measurement by MRI could be an important biomarker of degeneration like in the case of AD [19,20]. They also represent a tool for anti-amyloid vaccination studies [21,22]. Our results also confirm earlier works [15–18] that human chips can be used in non-human primates.

Among more than 14,000 genes, we have identified 695 genes (around 4.9%) with significant expression changes in young, aged and “AD-like” animals. Analysis of the cortex transcriptome from human brain samples at different ages indicates that the expression of about 400 genes among 11,000 (around 3.6%) is significantly changed [22]. Our results are in the same order of magnitude.

We then compared the list of genes that changed in the human frontal cortex sorted by Lu et coll. (2004) with the genes changing in the lemurian temporal cortex. We found 13 common genes (out of 150) and also 58 (out of 150) that belong to the same family and have similar biological function (e.g. VAMP1/VAMP2 or Synapsin II and synapsin IIb). Most of the genes that are differentially expressed in both works appeared to be involved in the synaptic transmission (8/15), Ca²⁺ homeostasis (5/10), cAMP signalling (2/2) and in transcription (6/8). In the study by Lu and coll, the expression of these genes was modified by age, whereas in our study, they are modified by age and/or by pathology.

The same applies to the fold changes that varied between 1.5 and 3. Moreover, analysis of the microarray data indicates that microarrays the overall rate of gene detection was about 20%. This value could appear to be rather low, but it has to be compared with other heterologous (Rhesus monkey with human microarrays) or homologous (human with human microarray) studies where only 30–35% and 30–40% of genes were detected ([18]; JDV, personal communication). Our results also confirm earlier works [15–18] that human chips can be used in non-human primates.

were up-regulated with age and down-regulated in “AD-like” animals (especially those involved in protein synthesis) belonged to the “metabolism and protein synthesis” module (Fig. 6A, module 3a). Nuclear factors (Fig. 6A, module 4a) were differentially expressed particularly in the “AD-like” group. Analysis of the data from the 2 females only showed that the general profiles were maintained when compared with the data from all animals (Fig. 6B). Nevertheless, the number of genes that were up-regulated with age was higher, whereas the number of down-regulated genes involved in signaling transduction (Fig. 6B, module 2b), metabolism (Fig. 6B, module 3a) and in epigenetic regulations (Fig. 6B, module 4e) was lower.
each group of animals (young adult, healthy old, and “AD-like”) has a distinctive gene expression profile in the temporal cortex, a structure involved in integrative functions such as memory. Previous studies have already concluded that the expression of many genes is modified during aging (reviewed in [2,23,24]). Specific profiles in relation with age were observed in human brain [22,25] and in Rhesus monkey brain, particularly in the corpus callosum [18] and in the frontal cortex [25]. Interestingly, each brain region of adult or healthy elderly subjects had a distinct gene expression profile [26–28]. Altered gene expression was also observed between healthy elderly individuals and AD patients, for review [29], particularly in the hippocampus [30,31] and in neurons of several brain structures [27].

Our results support the hypothesis that the events occurring during physiological brain aging and AD development are distinct. However, an important issue is to understand the transition from physiological to pathological brain aging which leads to neurodegenerative disorders. We observed that many of the genes up-regulated in healthy aged animals are involved in protein synthesis such as the genes coding for ribosomal proteins. These results support the idea that physiological aging, at least in the brain, is associated with permanent compensatory effects via increased protein synthesis. Conversely, these genes were down-regulated in the 2 “AD-like” animals, indicating a failure to compensate a decline in the metabolic activity in the brain of animals with amyloid deposits. Moreover, the expression of UBQLN1, an ubiquitin-like protein that plays a role in the regulation of the degradation of proteins by proteasome [32], was significantly decreased in both “AD-like” animals. This observation is important as accumulation of misfolded proteins, which are not degraded by the proteasome, may be involved in the development of neurodegenerative diseases. The expression of other genes that are related to the regulation of neural plasticity, an important process involved in brain aging [3,33], also was changed in “AD-like” animals in comparison to healthy old ones. Particularly, GRIK1, the NR1 subunit of the NMDA glutamate receptor, and GRIA1, a subunit of the AMPA glutamate receptor, which activate pathways involved in the induction of long-term potentiation (LTP) were markedly down-regulated. AD-related pathologic processes lead to synaptic loss and dysfunction, particularly of glutamatergic transmission [34,35] that can cause inhibition of LTP [36] as it has been shown also in rodents following acute treatment with Aβ oligomers [37]. Moreover, recent data have also demonstrated that changes in the expression of nuclear proteins, which induce epigenetic modifications of chromatin, play an important role in regulating synaptic plasticity and memory processes [38]. In our study, we observed that several genes implicated in epigenetic processes and transcription, such as histone acetyltransferases (HAT1, PCAF), histidine triad nucleotide binding proteins (HINT1, HINT2) and the cAMP responsive element binding protein (CREB1), were down-regulated during physiological and pathological brain aging. Conversely, KRAS and HBXAP, which mediates nucleosome assembly and chromatin remodeling, were up-regulated in healthy aged Microcebus murinus.

Our data also indicate that changes in gene profiles during aging in Microcebus murinus temporal cortex might show gender-specificity,
suggesting that brain continues to present sexual differences during the entire life. This observation needs, however, to be confirmed since both “AD-like” animals were females. Nevertheless, sexual dimorphism in gene expression in aging human brain has been reported by Berchtold et al. [28] and in primate brain by Reinius et al. [41], whereas Lu and colleagues [22] did not evidence significant gender differences in the transcriptomic profiles of the human frontal cortex.
Materials and Methods

Microcebus murinus

Figure 5. Transcriptional profiles in the temporal cortex of Microcebus murinus. Hierarchical clustering obtained with the 695 genes sorted by ANOVA. The transcriptional profiles of the 18 Microcebus murinus (i.e., 6 young adults (Yg), 10 old lemurs (Old) and 2 "AD-like" (AD)) showed 3 distinct regions: I- genes that are up-regulated in "AD-like" temporal cortex; II- genes that are down-regulated in "AD-like" temporal cortex and preferentially up-regulated in aged cortex; III- genes that are down-regulated in "AD-like" and preferentially up-regulated in young adult cortex. In red are shown genes that are over-expressed, in green genes that are under-expressed and in black genes without expression changes.

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Overall, our expression data support the relevance of Microcebus murinus as a valuable non-human primate model for studying molecular brain changes in relation with age and neurodegenerative diseases such as AD.

Immunohistochemistry

Presence of amyloid-b-42 (Aß42) peptide aggregates was assessed using the rabbit polyclonal FCA3542 antibody (Calbiochem, Merck-bio, Germany). Sections were deparaffinized and hydrated through ethanol gradient. After formic acid pretreatment, sections were pre-incubated with 10% goat serum in Tris-buffered saline for 30 min. Then, sections were incubated with anti-Aß42 antibodies (1:1000) at 4°C overnight. Immunological complexes were detected with biotinylated immunoglobulins (1:1000) and horseradish peroxidase-labelled avidin (Vector Laboratories, Burlingame, VT). Immuneactivity was revealed with 0.005% diaminobenzidine tetrahydrochloride (DAB, 0.35 mg/mL, Sigma, St Louis, MO). The primary antibody was omitted in the negative control and sections from Microcebus murinus brain specimens known to contain b-amyloid plaques were used as positive controls. The Microcebus murinus brain atlas was used to localize the brain area of each section [42].

RNA isolation and Affymetrix GeneChip processing

Total RNA was extracted from each temporal cortex sample (25–35 mg) using the RNeasy mini kit (Qiagen, Santa Clarina, CA) according to the manufacturer’s protocol. The quality of each purified RNA sample was checked with a NanoDrop Agilent BioAnalyzer (Agilent Technologies, Santa Clara, CA). An average of 2 µg of total RNA from each cortex sample was prepared for hybridization with Affymetrix HG-U133 Plus 2.00 GeneChip (Santa Clara, CA) according to the manufacturer’s protocol. This microarray contains 54,656 probes corresponding to 47,400 human transcripts. At the end of the experiment, the hybridized probes were scanned at a resolution of 3 µm in a confocal scanner (Affymetrix GeneChip Scanner 3000 7G). Affymetrix microarrays were processed in the Microarray Core Facility of the Institute of Research of Biotherapy, CHRU-INSERM-U11 Montpellier (http://irb.chu-montpellier.fr/).

Affymetrix GeneChip data analysis and filtering

Data were normalized with the GeneChip Operating Software (GCOS) algorithms. Then, the converted digital intensity values were stored as image data files and converted into cell intensity files using the Microarray Affymetrix Software 5.0 (MAS 5). MAS5 labels each transcript as “present” (P), “marginal” (M) or “absent” (A) by taking into account the average difference value calculated between the perfect match (PM) and the mismatch (MM) as detailed in the Affymetrix Genechip procedure (www. Affymetrix.com). A p-value risk was associated with the signal intensity. For each gene, the mean signal expression and standard deviation (SD) were calculated for each group. The fold change was determined by the ratio between old and young, and between
“AD-like” and healthy old in order to study gene expression during physiological and pathological aging, respectively. All primary expression data files (*.cel) and (*.xls) are available at the authors’ web site (http://www.mmdn.univ-montp2.fr/) and at GEO/NCBI (provisional accession number GSE21779). All steps were conducted according to the MIAME (Minimum Information About a Microarray Experiment) checklist [43].

Detection of genes that were differently expressed in the three groups

To identify genes differently expressed in relation with brain aging or pathology, filtered data were analyzed with the SAM (Significance Analysis of Microarrays) method [44], which uses permutations of repeated measurements to protect against non-normal data distribution. For that purpose, each group was compared to the other groups using 1,000 permutations. Because each animal may show intrinsic individual variability, the threshold for determining the rate of change was set at 1.5. SAM calculates the false discovery rate (FDR) with a q-value according to the delta cutoffs. The statistical significance of the genes was also investigated by Student t-test or by ANOVA with the “R” software. Finally, to construct a predictive model of the changes observed, a projection by principal component analysis (PCA) was carried out.

Gene profiling

The gene expression profile of each group was established using the Cluster and Treeview software programs [45]. These analyses

Figure 6. Gene changes in relation to their cellular function identified by gene ontology. The 695 genes sorted by ANOVA were classified into four main modules: 1- genes involved in brain plasticity [1a-neurotransmission, 1b-neurogenesis, 1c-adhesion and extracellular matrix, 1d-cytoskeleton]; 2- genes involved in transduction and signaling [2a-ion channels, 2b-kinases, 2c-phosphatases, 2d-transferases and 2e-growth factors]; 3- genes involved in metabolism and catabolism [3a-protein synthesis and maturation, 3b-proteolysis, 3c-glucolysis and lipid metabolism and 3d-mitochondrial metabolism]; 4- genes involved in nuclear activity [4a-nuclear factors, 4b-transcription regulation, 4c-cell cycle regulation, 4d-apoptosis and 4e- epigenetic control]. Red bars represent genes that are up-regulated and the green bars genes that are down-regulated in the temporal cortex of aging animals; hatched red bars represent genes that are up-regulated and hatched green bars genes that are down-regulated in the temporal cortex of “AD-like” lemurs. A. Classification with males and females together. B. Classification with females only. In aging animals genes involved in protein synthesis were more frequently up-regulated (red arrow), and the most important differences between aging and “AD-like” profiles concerned genes that have a role in protein synthesis and nuclear activity (blue arrows). The major differences between A and B are indicated by black arrows. doi:10.1371/journal.pone.0012770.g006
allowed us to build a hierarchical clustering of the genes that are significantly associated with brain aging.

Functional categorization of the identified genes
To categorize the genes that were differently expressed, we used two approaches; the first approach was consisting in searching the literature using PubMed (NCBI), GenBank (www.ncbi.nlm.nih.gov/GenBank/), or specific databases such as Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo/), the KEGG pathway database (www.genome.jp/kegg/pathway.html), UniProt (www.uniprot.org), and the Allen Brain Atlas (www.brain-map.org; http://humanconnectome.alleninstitute.org/has/human/docs.html). The second approach consisted in assigning genes to biological and molecular functions using the hierarchical database of the Gene Ontology (GO) consortium and EASE (Expression Analysis Systematic Explorer software; www.geneontology.org/). Finally, PathwayArchitect (Stratagene, Genome Exploration, UK) and IPA-Ingenuity (www.ingenuity.com) allowed us to build interactive networks.

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Supporting Information
Table S1 Genes sorted by anova
Found at: doi:10.1371/journal.pone.0012770.s001 (0.05 MB XLS)
Table S2 Gene functional categorization
Found at: doi:10.1371/journal.pone.0012770.s002 (0.19 MB XLS)

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Author Contributions
Conceived and designed the experiments: JMV GD. Performed the experiments: RAR SA VP NMF GD. Analyzed the data: RAR JRDV GD. Contributed reagents/materials/analysis tools: BM MP. Wrote the paper: JMV GD.
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