



Coordinated Gene Expression of Neuroinflammatory and Cell Signaling Markers in Dorsolateral Prefrontal Cortex during Human Brain Development and Aging

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Abstract

Background: Age changes in expression of inflammatory, synaptic, and neurotrophic genes are not well characterized during human brain development and senescence. Knowing these changes may elucidate structural, metabolic, and functional brain processes over the lifespan, as well vulnerability to neurodevelopmental or neurodegenerative diseases.

Hypothesis: Expression levels of inflammatory, synaptic, and neurotrophic genes in the human brain are coordinated over the lifespan and underlie changes in phenotypic networks or cascades.

Methods: We used a large-scale microarray dataset from human prefrontal cortex, BrainCloud, to quantify age changes over the lifespan, divided into Development (0 to 21 years, 87 brains) and Aging (22 to 78 years, 144 brains) intervals, in transcription levels of 39 genes.

Results: Gene expression levels followed different trajectories over the lifespan. Many changes were intercorrelated within three similar groups or clusters of genes during both Development and Aging, despite different roles of the gene products in the two intervals. During Development, changes were related to reported neuronal loss, dendritic growth and pruning, and microglial events; *TLR4*, *IL1R1*, *NFKB1*, *MOBP*, *PLA2G4A*, and *PTGS2* expression increased in the first years of life, while expression of synaptic genes *GAP43* and *DBN1* decreased, before reaching plateaus. During Aging, expression was upregulated for potentially pro-inflammatory genes such as *NFKB1*, *TRAF6*, *TLR4*, *IL1R1*, *TSPO*, and *GFAP*, but downregulated for neurotrophic and synaptic integrity genes such as *BDNF*, *NGF*, *PDGFA*, *SYN*, and *DBN1*.

Conclusions: Coordinated changes in gene transcription cascades underlie changes in synaptic, neurotrophic, and inflammatory phenotypic networks during brain Development and Aging. Early postnatal expression changes relate to neuronal, glial, and myelin growth and synaptic pruning events, while late Aging is associated with pro-inflammatory and synaptic loss changes. Thus, comparable transcriptional regulatory networks that operate throughout the lifespan underlie different phenotypic processes during Aging compared to Development.

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Introduction

The human brain changes structurally, functionally, and metabolically throughout the lifespan [1,2]. Programmed dendritic growth followed by pruning, neuronal loss, shifts in energy metabolism from ketone body to glucose consumption, and rapid myelination occur during development [3,4,5,6]. Many of these changes are completed within the first two decades of life, although myelination can continue into the fourth decade [5,7,8]. In middle age, the brain reaches a level of homeostatic stability, but neuronal

and synaptic loss associated with cognitive changes can appear later on [2,9,10,11,12]. Aging also is a risk factor for progressive brain disorders in which neuroinflammation plays a prominent role [13,14,15,16,17].

Neuroinflammation involves activation of resident brain microglia and astrocytes, and can be produced by different internal or external stresses [14,18,19,20]. Microglial activation *via* toll-like receptors (TLRs) or cluster of differentiation (CD)14 receptors releases cytokines such as interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF α) and interferon gamma (IFN γ),

chemokines such as fractalkine (CX3CL1), and nitric oxide (NO), following activation of inducible nitric oxide synthase (iNOS), thereby creating response cascades that can negatively impact brain structure and function [21]. Downstream activation of IL-1 receptors (IL-1R) and TNF α receptors on astrocytes and other cell types alters levels of transcription factors such as nuclear factor-kappa B (NF- κ B) and activator protein (AP)-2, which can increase expression of a number of inflammatory genes [21,22,23,24,25].

The term “inflamm-aging” has been proposed to describe the progressive increase in proinflammatory status in the brain with senescence [26]. Inflamm-aging may prime brain microglia and astrocytes to respond excessively to different stressors, including neurodegenerative, traumatic or infectious insults [27,28,29,30,31]. Increased inflammatory response markers with late-state brain aging have been documented in rodents [27,32], nonhuman primates [33], and humans [34]. Increases have been noted in proinflammatory cytokines IL-1 α , IL-1 β , IL-18 and IFN γ , major histocompatibility complex class II (MHC II), CD11b, scavenger receptors CD68, CD86 and CD40, and TLRs 1, 2, 4, 5, 7, and 9. In the human brain, increased TNF α and interferon gamma-inducible protein 16 (IFI-16) were reported, as were increased mRNA and protein levels of CD11b, glial fibrillary associated protein (GFAP), IL-1 β , iNOS, NF- κ B p50, cytosolic phospholipase A₂ (cPLA₂) Type IVA and cyclooxygenase (COX)-2, while levels of brain derived neurotrophic factor protein (BDNF) and synaptophysin (SYP) were reduced [34,35].

Cytokines, chemokines, growth and other microglial and astrocytic factors that change with age in the adult brain also have important regulatory actions during neurodevelopment [36,37]. For example, microglia participate postnatally in synaptic pruning and apoptosis, and produce nerve growth factor (NGF), BDNF, neurotrophin (NT)-3 and cytokines that influence neuronal path finding, synaptogenesis and experience dependent plasticity [38,39,40,41].

Multiple metabolic and protein networks have been described that underlie brain structure and function, and brain vulnerability to disease [42,43,44,45,46]. The extent to which these phenotypic networks or “cascades” are regulated at the transcriptional level, particularly during brain development, maturity, and aging are not well understood. To address this limitation, in the present study we analyzed age changes over the lifespan in brain mRNA levels of 39 genes whose protein products have been reported to be involved in neuroinflammation, synaptic integrity, neurotrophic effects, and related processes. As in our prior report on age expression of brain lipid metabolic markers [47], we employed the large-scale microarray dataset called BrainCloud, which contains genome-wide expression levels in frontal cortex from non-pathological individuals, in the fetal period to postnatal 78 years of age [48]. Similarly, and consistent with the literature, we considered gene expression in two distinct postnatal age intervals, Development (0 to 21 years) and Aging (22 to 78 years) [35,47,49,50], henceforth identified by capitalizations.

Based on prior studies, we hypothesized that expression of genes linked to neuroinflammation would increase with age in the Aging interval [29,34,51,52], while expression of genes coding for synaptic integrity and plasticity would decrease [9]. Furthermore, expression of these same genes would change during Development to reflect reported roles of their protein products in synaptic and neuronal growth, pruning, myelination, and other events in this period [1].

We also hypothesized that expression of genes coding for products that belong to common growth and neuroinflammatory cascades would be coordinately regulated during the lifespan, in relation to the specific phenotypic networks in which their proteins

interacted. Coordinated or synchronized gene transcription underlying changes in phenotype networks has been demonstrated in cell culture, rodent brain, and artificial systems [53,54,55], and in the human brain in relation to age [47,48,56,57,58].

Methods

BrainCloud

BrainCloud (<http://braincloud.jhmi.edu/>) is a publicly available software program that contains gene expression and methylation microarray datasets of more than 30,000 probes [48]. We analyzed data from 231 dorsolateral prefrontal cortex samples (Brodmann Areas 46/49) from subjects ranging in age from birth to 78 years. Results were transferred to an accessible and easily operated interface. Subjects had no history of significant psychiatric disorder, as determined by telephone screening and medical examiner reports [48]. Cause of death was listed as accident, homicide, or natural cause. Toxicology measurements were taken post-mortem and history of drug abuse and neuropathology tests were assessed [48]. Samples were genotyped with Illumina Infinium HD Gemini 1M Duo BeadChips or with Illumina Infinium II 650 K. mRNA was quantified with the Illumina Human 49K Oligo array (HEEBO-7 set) [48].

Based on the literature and to remain consistent with our prior publication [35,47,49,50], we divided our sample into a Development interval (0 to 21 years, n=87, mean age: 10.99) and an Aging interval (22 to 78 years, n=144, mean age: 45.38), capitalized to distinguish these defined intervals from the developmental and aging processes themselves. The brains were from 73 females and 158 males.

When multiple probes in BrainCloud represented a gene, we selected the probe sequence that covered all exon-coding regions of the gene's identified transcripts. The highest intensity probe (average log₂ intensity of fluorescent signal for this probe across all subjects) was taken when two probes covered all exon-coding regions of the transcripts. Most probes that met the first criterion also were the highest intensity probes of that gene. Genes were selected based on their availability in the BrainCloud dataset. Gene expression levels remained consistent with BrainCloud's calculated expression as log₂ (Sample/Reference), reference being the pooled RNA from all subjects [48].

Statistical Analyses

Pearson's r correlations were used to correlate expression levels of pairs of genes. Linear regression was used to relate expression to age within the separate Development and Aging intervals. Nonlinear best-fit comparisons with each gene were performed using ‘The extra sum-of-squares F test’ to compare one-phase decay to a first-order linear model. A four-way ANOVA that included relevant factors provided by BrainCloud software, such as gender, race and batch number, was performed to calculate fold-differences and p-values comparing the Aging with Development group. The p-values from the ANOVA results include correction of significance for multiple comparisons using the Benjamini-Hochberg [59] control of False Discovery Rate (FDR), also known as the “step-up” FDR procedure. Batch effect removal on factors (array batch, race, and sex) was performed using the sva package (version 3.8.0) in Bioconductor, based on the method of Johnson et al. [60]. No other normalization was performed on the raw data. Differential gene expression would be a result of age and not race, sex, or array batch. Pearson's r correlations and multiple ANOVAs were performed in Partek Genomics Suite (Version 6.6, Partek, St. Louis, MO, USA). Linear regression analyses and scatter plots were performed using GraphPad Prism version 6.0 for

Mac OS X (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

Heat Map

Heat maps, which are graphical representations of data where individual values are represented in a matrix on a color scale, were created using Partek Genomics Suite 6.6 to visualize correlated expression levels across all genes in the separate Development and Aging periods.

Results

Differences in Gene Expression during Development and Aging

Correlations with age. Table S1 presents the 39 genes that were selected in this study, with their corresponding chromosomal location, protein name, and reported general functions [61,62,63,64]. While many of their protein functions may be unrelated, many of their proteins have been reported to participate in phenotypic cascades involving cell growth, inflammation, synaptic maintenance and gene transcription, and other processes. For example, Figure 1, generated from the literature [65,66,67,68,69] and string interactions in GeneCards [64], illustrates several abbreviated cascades initiated by activation of TLR-2 and TLR-4 commonly found on microglia, and of IL-1R and TNF α receptors commonly found on neurons and astrocytes. These cascades are abnormal in a number of progressive neuroinflammatory diseases [13,15,69,70,71].

Of the genes listed in Table S1, Figure 2 summarizes those whose expression levels correlated significantly with age in the Development and/or Aging interval, and indicates by quadrant the direction of the correlation. Age-related expression of *MOBP* (myelin-associated oligodendrocyte basic protein) and of *NFKB1* increased during both Development and Aging (right upper quadrant), while expression of *NGF* and *CX3CLI* (Chemokine (C-X3-C Motif) Ligand 1) decreased during both Development and Aging (left lower quadrant). Expression of *SNCA*, coding for α -synuclein, increased during Development but decreased during Aging (right lower quadrant). Additionally, expression of *TRAF6* (TNF receptor associated factor 6), *PLA2G4A*, *PTGS1*, *TLR4*, *APP*, *MAP2*, and *IL1R1* increased while expression of *MYD88*, *DBN1*, *RELA*, *PDGFA*, and *IFI16* decreased during Development but not Aging. Conversely, expression of *GFAP*, *TSPO*, and *IL1RN* increased and of *PTGS2*, *BDNF*, *IL1RAP*, *SYP*, *CX3CRI*, and *NOS2* decreased during Aging, without changing significantly with age during the Development interval.

Figure 3 gives examples of genes whose expression levels correlated significantly with age during both the Development and Aging periods. Expression of *SNCA*, increased significantly with age during Development while declining during Aging. Expression of *MOBP* and *NFKB1* increased significantly with age during both intervals, while expression *CX3CLI* and *NGF* decreased. Expression of *CX3CRI*, and *PTGS2* (COX-2), declined with age whereas *GFAP* and *TSPO* increased during both intervals.

Visual observation of several gene expression levels during Development suggested non-linearity, with initial levels in the first year of life being higher or lower than later plateaus. To test this, we compared goodness of fit with a non-linear equation, $Y = (Y_0 - \text{Plateau}) * \exp(-K * A) + \text{Plateau}$ (where Y = expression level at age A , and Y_0 = expression level at $A = 0$ years) to that of a linear regression during Development for each gene studied. As illustrated in Figure 4, expression of *TLR4*, *IL1R1*, *NFKB1*, *MOBP*, *PLA2G4A*, and *PTGS2* increased in the first years of life

and reached a plateau, while expression of synaptic genes *GAP43* and *DBN1* decreased before reaching a plateau.

Mean expression level differences between Aging and Developmental Periods

As summarized in Table 1, mean expression levels were significantly (adjusted $p < 0.05$) lower during Aging than Development for *CX3CRI*, *CX3CLI*, *PTGS2*, *BDNF*, *CASP1*, *CD68*, *AIF1*, *MYD88*, *NGF*, *PDGFA*, *DBN1*, *IL1B*, and *SYP*, and significantly higher for *GFAP*, *TSPO*, *MOBP*, *TRAF6*, and *NFKB1* (highlights show significance after correction for multiple comparisons).

Correlated group expression changes during Development and Aging

Pearson's correlation matrices relating all combinations of the genes were visualized using unsupervised hierarchical clustering and heat maps within the Development (Figure 5a) and Aging (Figure 5b) intervals. Gene order based on hierarchical clustering are not conserved between Development and Aging heat maps, as they represent the highest probability of correctly clustering genes based on Pearson's r correlation in the individual intervals. In Figures 5a and 5b, genes that are highly positively correlated within a cluster are highlighted in green; those that are negatively correlated in red.

Three different clusters of genes whose expression levels were highly intercorrelated were identified in both the Development and Aging periods. As illustrated in Figure 5a, Development clusters were: Cluster 1: *GAP43*, *SYP*, *BDNF*, *IL1RAP*, *NOS2*, *MAPK14*, *MAP2*, *APP*, *SNCA*; Cluster 2: *BACE1*, *TRAF6*, *PTGS1*, *CX3CRI*; Cluster 3: *TSPO*, *TNFRSF1A*, *MYD88*, *IL1R1*, *NFKB1*, *AIF1*, *CD68*, *CASP1*, *TLR2*. As illustrated in Figure 5b, Aging clusters were: Cluster 1: *NOS2*, *CX3CLI*, *SYP*, *TRAP1*, *GAP43*, *BDNF*, *SNCA*; Cluster 2: *MAP2*, *APP*, *IL1RAP*, *PTGS1*, *CX3CRI*, *PLA2G4A*; Cluster 3: *GFAP*, *TSPO*, *TNFRSF1A*, *IL1R1*, *MYD88*, *CD68*, *AIF1*, *CASP1*, *TLR2*.

Figures S1a and S1b show pairwise correlation values between gene expression levels in the Development and Aging periods, respectively. Green coloring highlights statistical significance at $p \leq 0.0001$. The global clusters identified in Figures 5a and 5b, and the highly significant pairwise correlations illustrated in Figures S1a and S1b, show plausible synchronization of gene transcription within clusters or networks throughout the lifespan. The most significant pairwise correlations taken from Figures S1a and S1b, at $p < 10^{-10}$ and $r \geq |0.6|$, are given in Table 2.

Gene Expression and Chromosome Proximity

Four pairs of genes have the same band number on a chromosome: *PLA2G4A* and *PTGS2* (1q25); *IL1 β* and *IL1RN* (2q14); *CX3CRI* and *MOBP* (3p21.3); and *AIF1* and *MAPK14* (6p21.3) (cf. Table S1). At $p < 0.001$, expression levels of *PLA2G4A* and *PTGS2* were correlated positively during both Development and Aging, while expression levels of *IL1 β* and *IL1RN* were correlated positively during Development only. Expression levels of the other two gene pairs were not correlated significantly in either period.

Discussion

We used the BrainCloud database for human prefrontal cortex [48] to examine age variations in mRNA levels of 39 genes reported to be involved in pathways of neuroinflammation, cytokine signaling, arachidonic acid metabolism, neuronal and

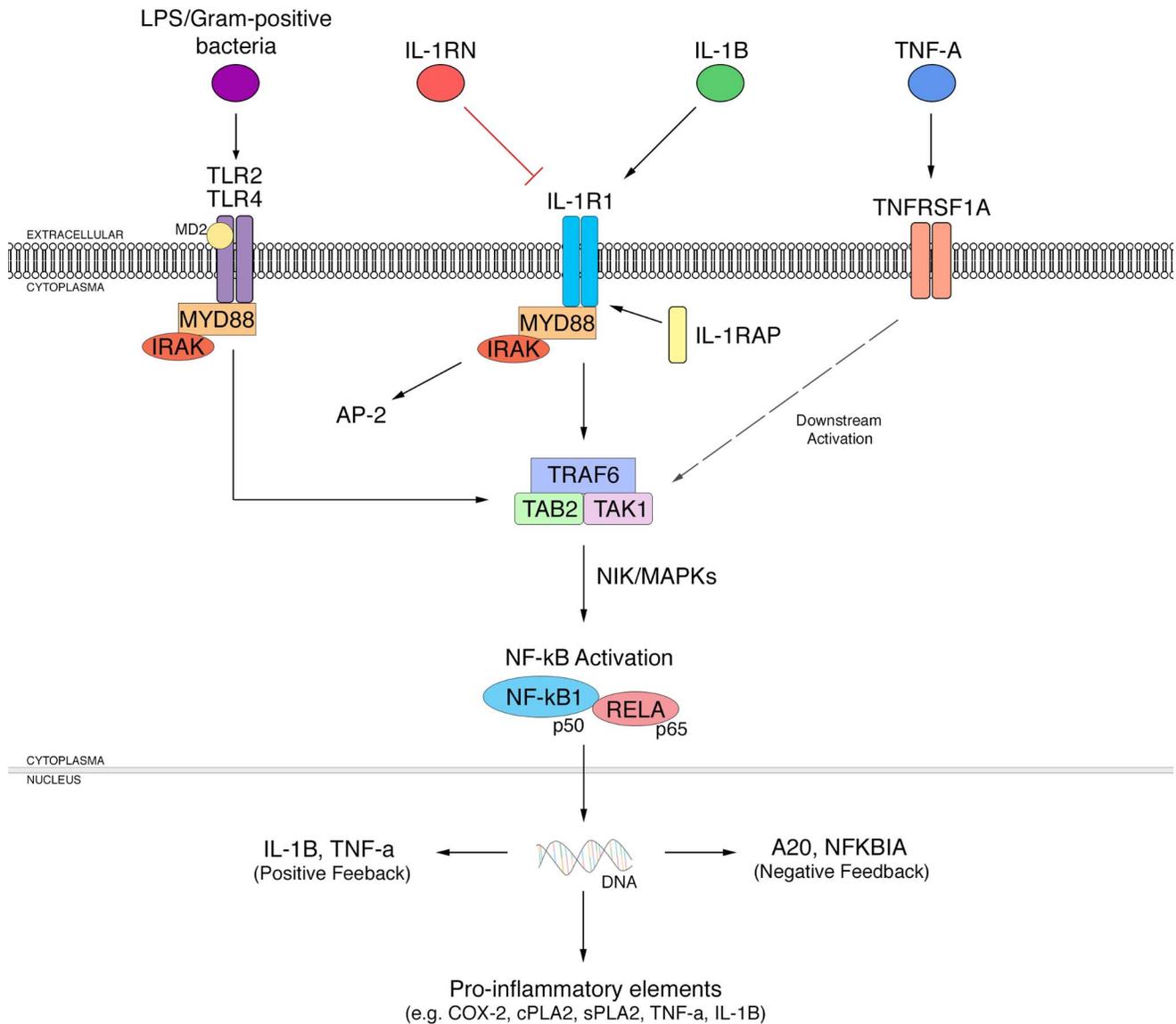


Figure 1. Pathways associated with activation of toll-like receptors TLR-2 and TLR-4, the IL-1 receptor IL-1R1, and the TNF α receptor, TNFRSF1A. IL-1 β binding to the IL-1R1 leads to recruitment of IL-1 receptor accessory protein (IL-1RAP), and can be blocked by the naturally occurring IL-1 receptor antagonist, IL-1RN. The IL-1R1/1 β /RAP signaling complex is capable of recruiting interleukin receptor-associated kinase (IRAK), IL-1 receptor accessory protein (IL-1RAP), and myeloid differentiation factor 88 (MYD88). IRAK can be phosphorylated and subsequently dissociate from the receptor complex to interact with tumor necrosis factor receptor-associated factor (TRAF6) and TGF- β activated kinase 1 (TAK1)/MAP3K7 binding protein 2 (TAB2) complex. The TLR2 and TLR4 cascades are simplified in Figure 1 into a single cascade. However, gram-negative bacterial lipopolysaccharide (LPS) can activate TLR4, which associates with lymphocyte antigen-96 (MD-2) while gram-positive bacteria are recognized by TLR2 [115]. Their activation effects converge with those of the IL-1R and TNF α receptor on the TRAF6 complex. IL-1R and all TLRs except TLR3 exhibit the same Toll/interleukin-1 receptor (TIR) region that allows recruitment of MYD88 upon activation [116]. Nuclear I kappa kinase (NIK) and various mitogen-activated protein kinases (MAPKs) can promote activation of cytoplasmic nuclear factor-kappa B (NF- κ B). Activated NF- κ B then can enter the nucleus of the cell to regulate transcription of various genes by binding to their promoter regions. RELA (p65) and p50, proteins in NF- κ B family; AP-2: transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha); A20: tumor necrosis factor, alpha-induced protein 3; NFKBIA: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; COX-2: cyclooxygenase-2; cPLA₂: phospholipase A₂ (cytosolic, calcium-dependent); sPLA₂: phospholipase A₂ (secretory). doi:10.1371/journal.pone.0110972.g001

myelin integrity, synaptic function, neurotrophic action, and related processes. We divided the postnatal lifespan into Development (0–21 years) and Aging (22 to 78 years) intervals, on the basis of reported distinct functional and structural brain changes in these periods [4,5,47,49,72].

Confirming this division, expression patterns and age correlations of many of the chosen genes frequently differed significantly

between the two intervals. Genes with higher expression during Aging include *TSPO*, associated with microglial activation and cholesterol transport [73,74]; *GFAP*, associated with glial activation [71]; *TRAF6*, associated with TNF α signaling; *MOBP*, associated with myelin integrity; and *NFKB1*, coding for NF- κ B, a major transcription factor of inflammatory genes involved in innate immunity [25]. More genes were expressed at lower levels

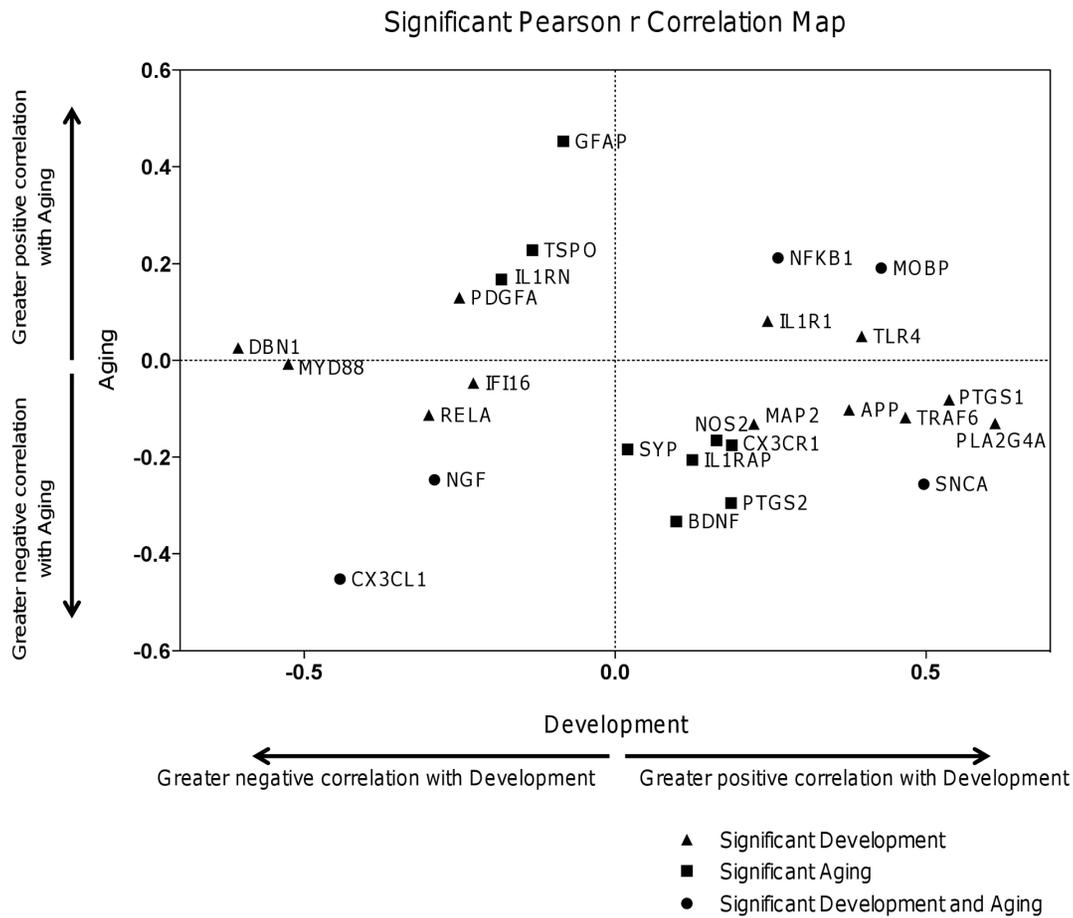


Figure 2. Statistically significant correlations with age in Development and/or Aging intervals. Graphical identification of genes with statistically significant ($p < 0.05$) Pearson r correlations in expression level during Development (x axis) and Aging (y axis) intervals. Gene expressions negatively correlated with age during Development are to the left, while genes positively correlated are to the right of the vertical line. Genes that were negatively correlated with age in the Aging group are below the horizontal line, while genes positively correlated are above line. Development: $n = 87$; Aging: $n = 144$.
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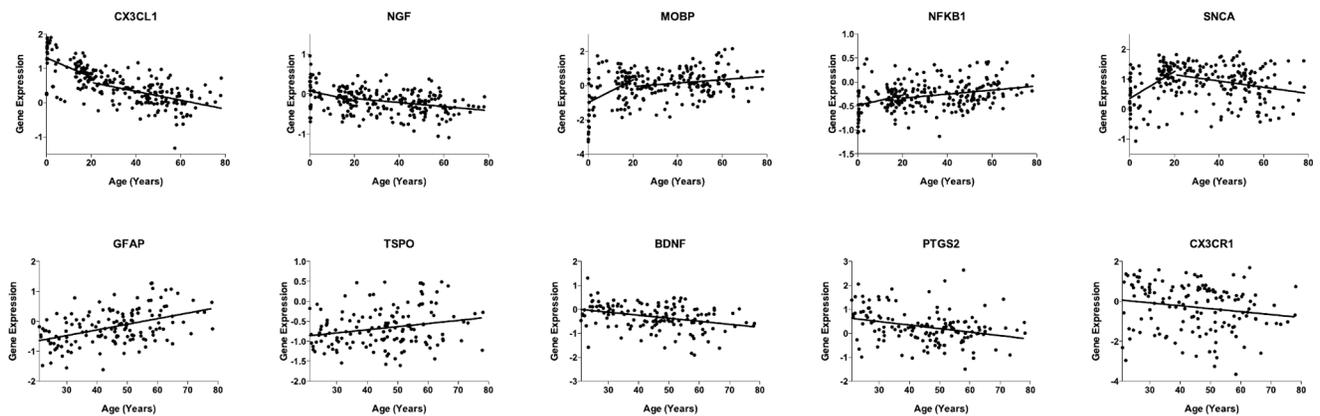


Figure 3. Significant linear regressions of gene expression during both Development and Aging intervals (top), and Aging interval alone (bottom). Scatterplots illustrating \log_2 gene expression over age in years. An increase or decrease of 1 on the \log_2 scale (y-axis) represents a two-fold change in gene expression in the positive or negative direction, respectively. Each data point represents observation from one brain (Development: $n = 87$; Aging: $n = 144$). Gene name (p-value during Development, p-value during Aging) - *CX3CR1* ($p < 0.0001$, $p < 0.0001$), *NGF* ($p = 0.006$, $p = 0.002$), *MOBP* ($p < 0.0001$, $p = 0.02$), *NFKB1* ($p = 0.01$, $p = 0.01$), *SNCA* ($p < 0.0001$, $p = 0.002$). Genes significant in only Aging interval - *GFAP* ($p < 0.0001$), *TSPO* ($p = 0.006$), *BDNF* ($p < 0.0001$), *PTGS2* ($p = 0.0003$), *CX3CR1* ($p = 0.03$).
doi:10.1371/journal.pone.0110972.g003

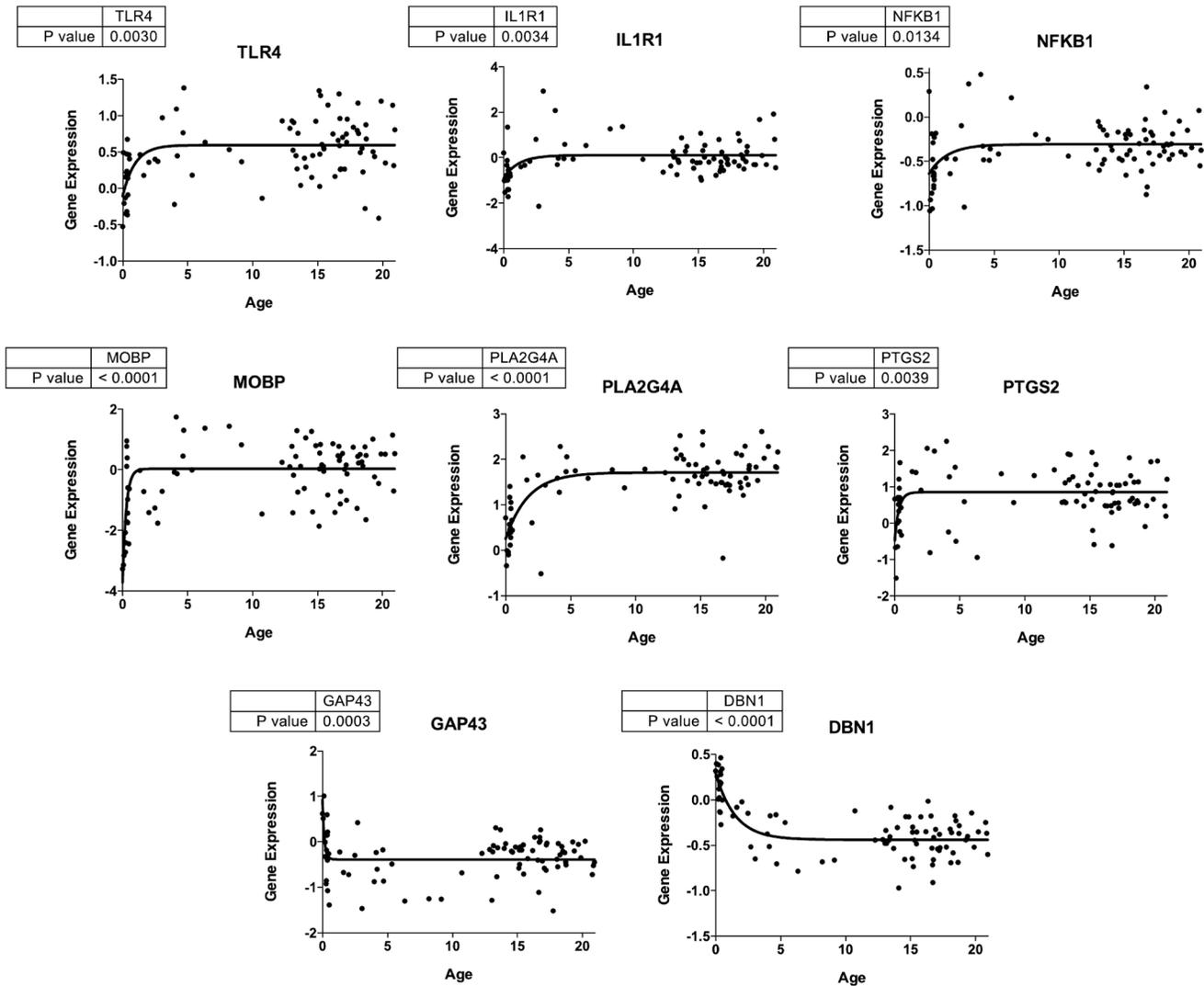


Figure 4. Nonlinear fits for expression levels with age of eight genes during Development. Fitted line added to expression data following equation for 0 to 21 years, $Y = (Y_0 - \text{Plateau}) \cdot \exp(-K \cdot A) + \text{Plateau}$, where Y = expression level at age A , and Y_0 expression level at $A=0$ years). An increase or decrease of 1 on the \log_2 scale (y-axis) represents a two-fold change in expression in the positive or negative direction, respectively. doi:10.1371/journal.pone.0110972.g004

during Aging than Development, reflecting a reduced intensity of early developmental events during Aging. These genes are related to synaptic integrity, neuronal growth, neurotrophic, glial changes and other development modifications (*CX3CR1*, *CX3CLI*, *PTGS2*, *BDNF*, *CASP1*, *CD68*, *AIF1*, *MYD88*, *NGF*, *PDGFA*, *DBN1*, *IL1B*, *SYP*, and *BACE1*).

Neurodevelopment is influenced largely by programmed transcriptional changes involving neuronal, glial and synaptic integrity, and myelination, whereas after 21 years of age, gene expression reaches a homeostatic state that depends more on factors such as health status, environment, and nutrition [48,57,75,76]. Late senescence becomes a risk factor for neurodegenerative diseases such as Alzheimer's and Parkinson's disease, when the brain shows increased inflammatory, apoptotic, and arachidonic cascade markers, but reduced neurotrophic and synaptic markers [9,13,15,77,78,79]. Our data in the Aging interval indicate many significant positive age correlations in expression of genes in the former category (*GFAP*, *TSPO*, *TRAF6*, *NFKB1*, *TLR4*, *IL1R1*) but decreased correlations for

genes in the latter category (*BDNF*, *SYP*, *SNCA*, *NGF*) (Figure 2). These findings support the proposition that aging increases vulnerability to neurodegenerative disease and is a priming factor for it [9,13,15,26,27,28,29,30,31,77,78,79].

The significant age changes in mRNA levels during the Aging interval do not always correspond to reported protein changes. $\text{TNF}\alpha$ and IFI-16 protein levels were reported to increase between 26 to 106 years in postmortem human brain [35], whereas we did not find age increases in *TNFRSF1A* or *IFI16* expression in the Aging interval (Figure 2), or on average between Aging and Development (Table 1). On the other hand, and consistent with our expression changes, positive age correlations between 42 and 70 years were reported in brain mRNA and protein levels of GFAP, IL-1 β , iNOS, NF- κ B p50, cPLA₂ IVA and COX-2, while levels of BDNF and SYP declined in this period [34]. Some of the changes correlated with promoter hypermethylation of *BDNF* and cyclic AMP responsive element binding protein (*CREB*), and hypomethylation of Bcl-2 associated X protein (*BAX*), suggesting epigenetic influence [80]. Another study also reported a decrease

Table 1. Statistically significant mean gene expression differences between Aging and Development Periods (Multiple ANOVA results).

Reduced with Aging			Increased with Aging		
Gene	Adjusted p-value	Fold-Difference	Gene	Adjusted p-value	Fold-Difference
<i>CX3CL1</i>	5.07E-21	-1.75	<i>GFAP</i>	4.33E-05	1.37
<i>CX3CR1</i>	8.00E-06	-2.00	<i>TSPO</i>	1.87E-03	1.23
<i>BDNF</i>	1.53E-05	-1.39	<i>MOBP</i>	2.77E-03	1.43
<i>CD68</i>	1.56E-04	-1.26	<i>TRAF6</i>	3.41E-03	1.17
<i>CASP1</i>	1.63E-04	-1.35	<i>NFKB1</i>	7.76E-03	1.11
<i>AIF1</i> (IBA1)	1.63E-04	-1.31	<i>TLR4</i>	6.16E-02	1.12
<i>PTGS2</i> (COX-2)	1.75E-04	-1.44	<i>IL1R1</i>	9.70E-02	1.21
<i>PDGFA</i>	1.34E-03	-1.14			
<i>DBN1</i>	1.34E-03	-1.13			
<i>MYD88</i>	1.34E-03	-1.24			
<i>NGF</i>	1.34E-03	-1.14			
<i>SYP</i>	2.11E-03	-1.18			
<i>IL1B</i>	7.33E-03	-1.22			
<i>BACE1</i>	5.33E-02	-1.11			

Multiple ANOVA results, giving fold changes, showing that mean expression was significantly lower (left) or higher (right) in Aging compared with Development. A negative fold-change represents decreased expression in the Aging compared to Development group, and *vice versa*. Italicized p-values are nonsignificant after correction for multiple comparisons. In p-values, term E-number = $\times 10^{-\text{number}}$. (Development: n = 87; Aging: n = 144). doi:10.1371/journal.pone.0110972.t001

in BDNF protein during the Aging interval [81], while another reported decreased protein levels of DBN1, GAP-43, and SYN [9]. In general agreement, we found significant age increases in expression of *GFAP*, *IL1R1*, and *NFKB1*, and reductions in expression of *BDNF* and *SYP* (Table 1).

Age correlations often differed between the Development and Aging intervals, indicating different roles for the gene products over the lifespan (Figures 2 and 3, Table 1). Expression of *SNCA* increased during Development but decreased during Aging; expression of *PLA2G4A*, *PTGS1*, *TRAF6*, *TLR4*, *APP*, and *IL1R1* increased and expression of *DBN1*, *MYD88*, *RELA*, *PDGF*, and *IFI16* decreased during Development alone. Expression of *MOBP* increased during both intervals, consistent with continued myelination into the fourth decade in frontal cortex [5], as did expression of *NFKB1*, while expression of *NGF* decreased during both periods, suggesting reduced neuroplasticity [82]. The NF- κ B system can be stimulated by a number of cell surface receptors (Figure 1), as well as by oxidative stress, hypoxia, and genotoxic stress [25]. In non-stimulated cells, NF- κ B complexes are bound in cytoplasm to inhibitory I-kappa-B (I κ B) proteins. Stimulation phosphorylates I κ B proteins, which are ubiquitinated and broken down, allowing the NF- κ B complex to enter the nucleus and activate transcription of multiple genes, particularly related to inflammatory cascades [25].

In the first six months of life, a number of genes showed non-linear expression changes that later reach a plateau (Figure 4). In this same period, neuronal density in layers 2–3 of human frontal cortex falls by 80% [3]. However, dendritic spine densities at different levels of prefrontal cortical pyramidal neurons rise from birth to about 5–10 years of age, and then decline [56,83]. Metabolic changes also occur, as the brain shifts from using ketone bodies to glucose in the first months of life [6]. Expression of *GAP43* and *DBN1*, coding for presynaptic GAP43 and postsynaptic dendritic spine drebrin, decreased nonlinearly. As dendritic spine density increases in the first 5–10 years (see above), *DBN1*

and *GAP43* likely change in this period. Expression increased for *PLA2G4A* and *PTGS2* coding for postsynaptic functionally-coupled cPLA₂ Type IVA and COX-2 [84,85,86] suggesting a growing role for arachidonic acid signaling in neurotransmission at dendritic spines [87,88].

Increases in *TLR4* and *IL1R1* in the first years of life may reflect increased receptivity of microglia, on which these receptors are located [89], as microglia participate postnatally in synaptic pruning and apoptosis [38,39,40,41]. In this regard, higher (2-fold) levels of expression of *CX3CR1* (chemokine (C-X₃-C motif) receptor 1) and of its ligand fractalkine (*CX3CL1*) during Development than Aging highlight the importance their protein products in early neuronal-glial interactions. *CX3CR1* is expressed exclusively by microglia in brain [90], whereas *CX3CL1* is highly expressed in neurons. Knocking out *CX3CR1* reduced neuron loss [91] and amyloid-beta deposition [92] in Alzheimer's disease mouse models, and interfered with formation of thalamocortical synapses during development, when fractalkine is overexpressed.

Gene expression clusters in the heat map matrices of Figures 5a and 5b identify genes having high intercorrelated expression patterns as the brain ages. The clusters were similar in the Development and Aging intervals. Thus, comparable transcriptional regulatory networks operate throughout the life span, but underlie different phenotypic processes during Aging compared to Development. Gene products within Cluster 1 of both Development and Aging (*GAP43*, *SYN*, *BDNF*, *NOS2*, *SNCA*) are involved in synaptic signaling and integrity, cellular stress, and neurogenesis. Gene products in Cluster 2 of both periods (*BACE1*, *CX3CR1*, *PTGS1*, and *PLA2G4A*) are involved in the arachidonic acid cascade, protease activity, APP processing, and inflammatory processing. Gene products in Cluster 3 of both groups (*TSPO*, *TNFRSF1A*, *IL1R1*, *MYD88*, *CD68*, *AIF1*, *CASP1*, and *TLR2*) are involved in microglial, inflammasome, NF- κ B signaling, and various neuroinflammatory responses.

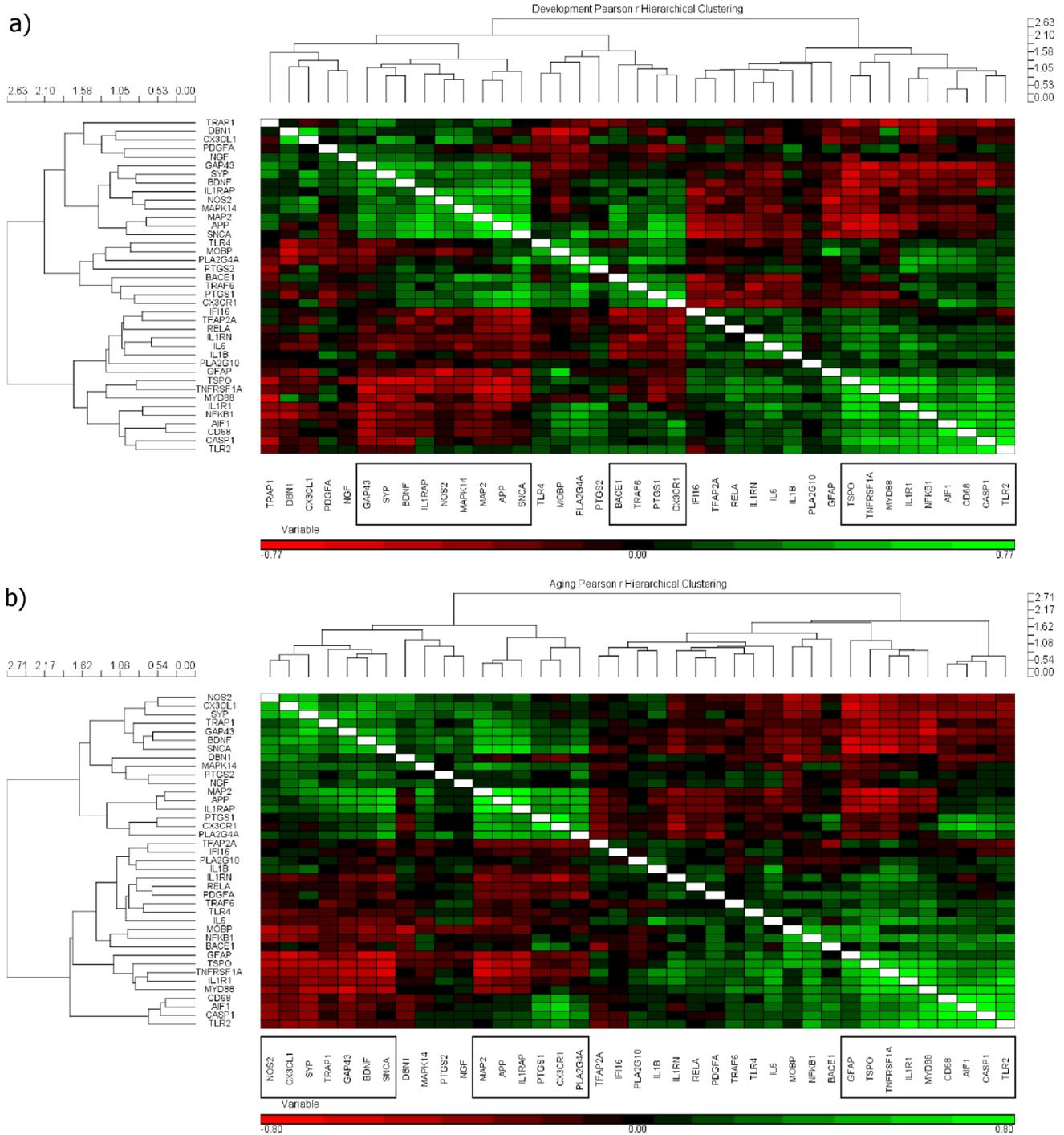


Figure 5. Similarity matrices (hierarchically clustered heat maps) of Pearson's r correlations of gene expression levels with age in Development (A) and Aging (B) groups. Red indicates negatively correlated associations; green are positively correlated associations, while black represents non-significant associations between gene pairs. Genes are clustered hierarchically along the left y-axis, which is mirrored above in each heat map. doi:10.1371/journal.pone.0110972.g005

Pairwise correlations, whether positive or negative, are more frequently significant during the Aging than Development interval (Figures S1a and S1b, Table 2). This may reflect our selective choice of genes, but if confirmed would suggest a more stable state of synchronized gene expression in the Aging than Development interval. This is likely since genome-wide promoter DNA

methylation of CpG dinucleotides in human prefrontal cortex changes less in adulthood than early childhood [57]. Mean cortical global methylation is increased in the Aging interval, which may reflect some gene silencing [57], but global methylation is higher in late senescence [34].

Table 2. Highly significant ($r \geq |0.6|$) pair-wise correlations in age-related gene expression during Development and Aging intervals.

Development				Aging			
Gene1	Gene2	Correlation	p-value	Gene1	Gene2	Correlation	p-value
MAP2	APP	0.77	1.53E-18	MAP2	APP	0.80	5.61E-34
AIF1	CD68	0.75	3.86E-17	IL1R1	TNFRSF1A	0.76	3.38E-29
TLR2	CASP1	0.67	8.13E-13	AIF1	CD68	0.75	1.49E-27
APP	SNCA	0.67	9.95E-13	AIF1	CASP1	0.71	4.82E-24
MOBP	DBN1	-0.63	3.20E-11	TLR2	CASP1	0.69	1.84E-22
MAP2	SNCA	0.63	3.37E-11	CD68	CASP1	0.68	1.54E-21
TSPO	MYD88	0.63	5.75E-11	CX3CR1	PTGS1	0.68	1.68E-21
IL1R1	NFKB1	0.61	2.88E-10	TLR2	IL1R1	0.68	3.63E-21
CX3CR1	PTGS1	0.60	4.02E-10	IL1RAP	APP	0.67	1.25E-20
MYD88	CASP1	0.60	6.18E-10	IL1RAP	MAP2	0.67	1.87E-20
IL1R1	TNFRSF1A	0.60	7.41E-10	TSPO	TNFRSF1A	0.66	6.52E-20
				TSPO	MYD88	0.66	6.98E-20
				TLR2	CD68	0.66	1.16E-19
				AIF1	CX3CR1	0.66	2.20E-19
				TNFRSF1A	MYD88	0.65	5.94E-19
				AIF1	TLR2	0.65	1.12E-18
				TSPO	IL1R1	0.64	2.63E-18
				TSPO	GFAP	0.63	1.26E-17
				MAP2	SNCA	0.63	1.64E-17
				TLR2	MYD88	0.62	3.05E-17
				TSPO	MAP2	-0.60	7.67E-16
				MYD88	CASP1	0.60	1.10E-15
				IL1R1	MYD88	0.60	1.16E-15
				TSPO	SNCA	-0.60	1.41E-15

In p-values, term E-number = $\times 10^{-\text{number}}$.
doi:10.1371/journal.pone.0110972.t002

Since the subjects were considered to be healthy, some presumably deleterious expression changes in the Aging interval had not progressed sufficiently to produce noticeable functional deficits, although they may have increased vulnerability to stress and other disease factors. Upregulated translator protein (*TSPO*) has been imaged using positron emission tomography in patients with mild cognitive impairment (MCI) and Alzheimer's disease [73,93,94]. Thus, the higher *TSPO* expression in Aging than Development is consistent with late-stage "inflamm-aging" in presumably healthy subjects (Table 1) [26]. In support, expression levels of *TRAF6*, *NFKB1*, *TLR4*, and *IL1R1*, important in the initial inflammatory response involving NF- κ B (Figure 1), and of *GFAP*, were upregulated in the Aging interval. Nevertheless, not all gene markers of inflammation or microglial/astrocytic activation were upregulated. Expression levels of inflammation-related genes *CASP1*, *PTGS2*, *MYD88*, and *IL1B*, and of microglia genes *CD68* and *AIF1* (also known as IBA1), were reduced in the Aging group.

Our age correlations identified unexpected relations between different genes. Highly significant pairwise correlations were found between expression levels of *APP* and *MAP2*, of *AIF1* (IBA1) and *CD68*, and of *IL1R1* and *TNFRSF1A*. *APP*, a component in Alzheimer's disease pathogenesis, normally helps to maintain functional synapses [95]. Microtubule-associated protein 2 (MAP2) is found in post-synaptic dendrites and is functionally similar to tau protein, whose abnormal phosphorylation is another key component in Alzheimer's disease. *AIF1* (IBA1), expressed in macrophages and microglia, and *CD68*, expressed in macrophages and monocytes, contribute to the inflammatory response in brain. Also, *IL1R1* and the TNF α receptor (*TNFRSF1A*) are important in initial signaling in inflammation (Figure 1). Among the other highly correlated genes, *CASP1* is involved in inflammasome formation [96], and *TSPO* is upregulated during neuroinflammation. Disturbed α -synuclein (*SNCA*) and *APP* occurs in Parkinson's disease and Alzheimer's disease respectively, and expression of *SNCA* and *APP* was very highly correlated in Development ($r=0.67$) and in Aging ($r=0.56$) (Figure S1). The Aging interval had a greater number of significant correlations between genes, and many significant correlations occurred consistently in Development and Aging. The correlated expression of genes in the canonical pathway of NF- κ B (*NFKB1*, *MYD88*, *TLR4*, *IL1R1*, *TRAF6*) also showed a highly integrated network of genes with similar expression patterns with age [25].

Significant correlations in gene expression that corresponded to chromosome proximity (Table S1) for *PLA2G4A* (cPLA₂ IVA) and *PTGS2* (COX-2) at locus 1q25, and for *IL1R1*, *IL1B*, and *IL1RN* at locus 2q14 indicate robust co-regulatory elements and possible coevolution [47,97,98,99]. The IL-1 receptor type 1 (*IL1R1*), a key cytokine receptor in innate immunity, interacts with *IL1B* and the IL-1 receptor antagonist (*IL1RN*) in a complex cascade (Figure 1) [36]. During Development, expression of *IL1B* and *IL1RN* exhibited a strong positive correlation ($p<0.001$), but their expression was not correlated significantly during Aging. Increased IL-1R signaling has been implicated in bipolar disorder, Alzheimer's disease, and HIV-1 encephalopathy [100,101,102]. Our changes during Development and Aging are consistent with neuroregulatory as well as neuroinflammatory functions involving the NF- κ B transcription system.

Some of the expression changes with senescence in this study correspond to changes reported in postmortem frontal cortex from Alzheimer's disease, bipolar disorder, and schizophrenia patients, compared to age-matched non-pathological cortex. Alzheimer's disease cortex showed significantly increased mRNA and protein levels of IL-1 β , TNF α , GFAP, CD11b, cPLA₂ IVA sPLA₂ IIA

COX-1 and COX-2 [13,103,104], but decreased levels of pre-synaptic synaptophysin (SYP) and post-synaptic drebrin (DBN1) [9,13]. In bipolar disorder, protein and mRNA levels of neuroinflammatory markers (IL-1 β , IL-1R, MYD88, NF- κ B1) and of activated microglia and astroglial markers (GFAP, NOS2, c-Fos, and CD11b) also were significantly higher than in control cortex [69,105]. These changes were accompanied by reduced expression of anti-apoptotic factors B cell lymphoma (Bcl)-2, BDNF, SYN, and DBN1, but increased expression of pro-apoptotic Bax, BAD, and active caspase (CASP)-3 and CASP-9 [15]. Ca²⁺-dependent cPLA₂ IVA, secretory sPLA₂ IIA and COX-2 also were overexpressed [70]. Similar changes were noted in schizophrenic frontal cortex [71].

This study has several limitations. Selection criteria for the microarray gene probes were a way to biologically standardize all probes by their protein-coding regions. There are other criteria of selection; however, we found that taking mean expression data of all probes for a given gene was not a good representation of gene's expression (data not shown), as expression levels differed between probes of the same gene.

BrainCloud contains expression data selective to the prefrontal cortex [48]. The prefrontal cortex shows prolonged development and preliminary degradation associated with aging earlier than other brain regions [5,106,107]. Other studies have found differential gene expressed based on cell type, such as the Allen Brain Atlases and the Loerch study; however, BrainCloud did not differentiate between cell types [108,109,110]. Favorably, BrainCloud has a large number of samples ($n=269$) compared with other aging databases with time points through a lifespan. For further discussion on BrainCloud and its application, see publications [47,48].

In the future, it would be of interest to consider mechanisms underlying the age-related expression changes. These may involve histone acetylation and methylation, transcription factors, miRNAs, DNA sequences of cis-elements (transcription factor binding sites), all of which can influence mRNA expression [57,111,112,113]. In this regard, many genes whose expression decreases with age appear to have higher promoter GC content than other genes [49], suggesting differences in methylation state, and human brain aging is associated with changes in global methylation [34,57]. As we considered only two transcription factors in this study, *TFAP2A* (AP-2) and *NFKB1* (NF- κ B), future aging studies may consider more.

Supporting Information

Figure S1 Matrices showing Pearson correlation coefficients between expression levels of individual gene pairs during Development (A) and Aging (B). Green highlights coefficients that are significant at $p<0.0001$. Hierarchy in gene order corresponds to hierarchy in Figures 5A and 5B. (XLSX)

Table S1 Selected genes, chromosomal locations, protein description, and major reported functions. Based on the literature, genes whose protein products participate in major functions may be categorized as follows: (1) Synaptic function, *SYP*, *DBN1*, *SNCA*; (2) Growth and maintenance, *BDNF*, *NGF*, *GAP43*, *PDGFA*; (3) Myelin integrity, *MOBP*; (4) Neuroinflammation: (a) Microglial activation, *CD68*, *TSPO*, *TLR4*, *TLR2*, *NOS2*, *AIF1*; (b) Cytokine and chemokine processes, *CX3CR1*, *CX3CLI*, *IFI16*, *IL1B*, *IL1R1*, *IL1RN*, *IL1RAP*, *IL6*, *TNFRSF1A*, *TRAF6*, *TRAP1*, *MAPK14*, *MYD88*; (c) Glial activation, *GFAP*; (d) Apoptosis, *CASP1*; (5) Arachidonic acid cascade, *PLA2G4A*, *PLA2G10*, *PTGS1*, *PTGS2*; (5) Amyloid

membrane processing, *APP*, *BACE*; (6) Microtubules, *MAP2*; (7) Transcription factors, *NFKB1*, *TFAP2A* [61,62,64,114]. (DOCX)

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Author Contributions

Conceived and designed the experiments: CP VR MC SR. Performed the experiments: CP. Analyzed the data: CP VR. Contributed reagents/materials/analysis tools: JR KA HM. Wrote the paper: CP SR.

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