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Large-scale estimates of cellular origins of mRNAs: Enhancing the yield of transcriptome analyses

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Abstract

Gene expression profiling holds great promise for identifying molecular pathologies of central nervous system disorders. However, the analysis of brain tissue poses unique analytical challenges, as typical microarray signals represent averaged transcript levels across neuronal and glial cell populations. Here we have generated ratios of gene transcript levels between gray and adjacent white matter samples to estimate the relative cellular origins of expression. We show that incorporating these ratios into transcriptome analysis (i) provides new analytical perspectives, (ii) increases the potential for biological insight obtained from postmortem transcriptome studies, (iii) expands knowledge about glial and neuronal cellular programs and (iv) facilitates the generation of cell-type specific hypotheses. This approach represents a robust and cost-effective "add-on" to transcriptome analyses of the mammalian brain. As this approach can be applied *post hoc*, we provide tables of ratios for analysis of existing mouse and human brain datasets.

Keywords: mRNA; Array; Transcriptome; White matter; Gray matter; Mouse; Human

1. Introduction

DNA microarrays monitor differences in RNA abundance between samples which are assumed *a priori* to represent differences in cell function or activity. This unbiased information on expression levels of tens of 1000 of genes potentially identifies new genes and pathways that correlate with brain dysfunction. In particular, gene expression profiling studies have already started to yield valuable information on possible mechanisms of neuropsychiatric disorders (Mirnics et al., 2006). However, human postmortem samples typically contain all cellular populations present in gray matter (GM), including neurons, interneurons, glial and endothelial cells. As gene transcript levels represent averaged values across cells with distinct transcriptional programs, array results cannot be directly attributed to any particular cell type, with the exception of known cell class-specific markers. The possibility of extracting homogeneous cell types with laser-capture microscopy potentially addresses this concern; however, differences in experimental design and technical limitations in sample collection and quantity of harvested RNA makes this approach more appropriate to targeted follow-up studies based on candidate cellular populations.

Here we show that ratios of transcript levels between GM and adjacent white matter (WM) samples can be used as estimates of relative glial to neuronal origins of transcripts for genes within GM samples. Of special interest to the growing field of transcriptome analysis of brain function, we describe several examples of analyses where combining large-scale information about WM/GM ratios with traditional investigations of transcriptomes provided additional and new analytical perspectives that go beyond up- or down-regulation of individual transcripts, and that facilitated the development of cell-type specific hypotheses of brain dysfunction.

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2. Methods

2.1. Cohorts and samples

Datasets previously generated by our group in two human postmortem cohorts and one mouse cohort were used here. Cohort 1 included postmortem brain samples from 39 human subjects. Brodman areas 9 (BA9) and 47 prefrontal cortex (PFC) GM samples were collected. Subject description, array parameters and data from cohort 1 were previously described (Erraji-BenChekroun et al., 2005; Galfalvy et al., 2003; Sibille et al., 2004). Cohort 2 included 34 subjects from the University of Pittsburgh Brain Donation Program and collected from the Allegheny County Medical Examiner's Office (see Supplemental information). Anterior cingulate gyrus (ACG) and amygdala (AMY) GM samples were obtained in the context of a study of major depression (16 major depression subjects and 18 control subjects). All subjects were male and died relatively rapidly without a prolonged pre-agonal phase. Average age (S.D.) at time of death was 52.0 ± 10.7 years old. Mean (S.D.) values for brain technical parameters were consistent with excellent preservation of RNA quality: postmortem interval (15 ± 5 h), brain pH (6.83 ± 0.21) and RNA quality (Agilent Bioanalizer RNA integrity number, RIN = 8.2 ± 0.5). Cohort 3 included 30 adult male BalbC mice. Frontal cortex (FC), AMY and dentate gyrus (DG) of GM samples were collected in the context of a study on stress and antidepressant treatment in a mouse model of depressive symptoms. Additional information on GM results from cohorts 2 and 3 datasets will be provided with the publication of two reports on the molecular correlates of depression in cohort 2 and on the transcriptional effects of chronic mild stress and antidepressant treatments in the mouse cohort 3 (in preparation). WM samples were collected adjacent to GM samples in human subjects. To collect array samples, frozen blocks were sectioned with a cryostat at 200 μm. White matter was separated with a clean scalpel blade from the gray matter samples at the clearly visible boundary with cortical layers and stored in separate tubes. ACG samples were collected in the rostral subgenual part of the cingulate gyrus, thus, corresponding WM samples also contained RNA material from the corpus callosum that is immediately adjacent. Amygdala white matter was collected at an easily recognizable thin band that is located between the lateral and ventral borders of the amygdala and the adjacent cortex. Mouse gray and white matter samples were dissected under microscope on RNAlater (Ambion Inc., Austin, TX, USA) protected 1 mm thick brain slices. For mouse WM, corpus callosum and anterior commissure were micro-dissected. WM samples ($n = 3$–7) were collected from randomly chosen control subjects in each cohort.

2.2. Microarray samples

Samples were processed as previously described (Galfalvy et al., 2003) and hybridized on U133A (cohort 1), U133-2.0 (cohort 2) and MOE430-2.0 (mouse) arrays (Affymetrix Inc., USA). Quality control measurements for RNA samples and arrays (Table 1) denoted high quality array sampling and hybridization that are consistent with robust and homogeneous datasets (See also Galfalvy et al. (2003), for array details on cohort 1). Array data were extracted and normalized with the robust multi-array algorithm (RMA) (Irizarry et al., 2003). For the purpose of this report, genes with signal below 50 in WM and GM samples were removed, leaving ~50–60% of genes for analysis. Note that the approach described in this report is flexible and highly similar results were obtained with lower or higher thresholds (see Section 4).

2.3. WM/GM ratios

For every gene in each brain area, two average signal intensities were calculated: one average value using all GM samples collected in that area and one average value using all WM samples collected nearby (see below). A ratio was then calculated between these two average values for each gene, representing the WM/GM ratio of gene transcript for that gene in that particular brain area. BA9 and BA47 GM signals were compared to the averaged value of 3 PFC WM samples. ACG and AMY were compared to distinct WM samples collected adjacent to the areas of interest (ACG, seven WM samples; AMY, four WM samples). In mice, three brain areas were compared to a unique set of seven WM samples. Details about WM sample collection are in Section 2.1.

Table 1
Microarray quality control parameters

	Species array					
	Cohort 1 human U133A 2.0		Cohort 2 human U133 Plus 2.0		Cohort 3 mouse MOE430 Plus 2.0	
	Average	S.D.	Average	S.D.	Average	S.D.
No. of arrays	85		74		96	
Background	38	19	46	7	52	12
Noise (RawQ)	2.97	0.77	1.53	0.28	1.83	0.47
Scale factor	1.16	0.38	2.35	0.95	1.27	0.52
P call	52.9	3.2	47.2	1.8	57.4	3.5
3′/5′ Actin	1.3	0.38	2.75	0.76	1.28	0.14
3′/5′ GAPDH	1.03	0.19	1.19	0.17	0.86	0.05

Array numbers are from all areas combined and include WM samples.

Although microarray datasets contained samples from psychiatric and control subjects (cohorts 1–2), and from treated and control mice (cohort 3), WM/GM ratios reported here (and in the Supplemental information) were generated using samples from controls only. Additional WM/GM ratios were also calculated using samples from psychiatric subjects or treated mice for the purpose of some of the analyses described in this report. These WM/GM ratios were essentially identical to ratios generated in control samples, as Pearson correlation factors between ratios obtained between control ratio and "all samples" ratios were greater than 0.99 (see Section 3).

Using these ratios to estimate the relative cellular origins of expression, we separated genes into three categories, using an arbitrary 50% level of enrichment: genes whose transcripts are enriched glial populations (WM/GM > 1.5), gene transcripts that are enriched in neurons (WM/GM < 0.67; a 0.67 ratio correspond to a −1.5-fold change) and genes that are expressed in both cellular populations 0.67 < WM/GM < 1.5).

2.4. WM/GM-based analyses

2.4.1. WM content of individual samples

To assess variations in WM content across different samples, we measured the differences in WM/GM ratios for the 100 most glial enriched genes, compared to the averaged values for the whole group. The number of enriched genes (100) was arbitrarily chosen and can be adjusted at will. This approach is based on the assumption that more or less WM content within a particular GM sample would be consistently reflected in relative increase or decrease in WM/GM ratios for highly enriched WM genes within this sample. This variable WM content was characterized by calculating the relative deviation in WM-related signals for any sample compared to the group averaged values:

$$\text{deviation}\,(\%) = \text{average}_{\text{k-i}(1-100)} \times \left[\frac{\text{WM/GM}_{\text{sample k,gene i}}}{\text{average}(\text{WM/GM}_{\text{all samples,gene i}})}\right] \times 100$$

where k is a sample in the cohort and i represent WM-enriched genes.

2.4.2. Functional analysis

Rather than analyzing genes one at a time, gene functional class scoring (Pavlidis et al., 2002) gives scores to classes or groups of genes, representing the overall effect of age on these groups of genes. Genes with white to gray matter (WM/GM) changes <−1.5-fold were removed from the dataset to analyze glial-enriched functions, while genes with WM/GM changes >1.5-fold were not used for analyzing neuronal-enriched functions, leaving 8064 and 8585 genes, respectively. Gene ontology groups (Ashburner et al., 2000) with >200 or <8 genes were screened out, leaving 897 groups for glial-enriched and 970 for neuronal-enriched analysis (88% overlap). GO groups were scored as described (Pavlidis et al., 2002, 2004), using age-related *p*-values as gene scores. See details about analysis and results in Erraji-BenChekroun et al. (2005).

2.5. Statistical analysis

The methods described in this report are for the most part exploratory and designed to help generate new hypotheses based on large-scale gene expression data. Since different numbers ($n = 3$–7) of WM samples had been collected in the three cohorts, statistical thresholds would yield different distributions across cohorts. Importantly, the particular strength of the WM/GM approach is that findings rely on converging results across a large number of genes (see Section 4). Thus, we have used fold changes for selecting WM- or GM-related genes, with arbitrary cut-off values at 50% of enrichment. These cut-off values can easily be adjusted up or down, as WM/GM ratios form a continuous distribution (Fig. 3A). Of course, these cut-off values can also be replaced by statistical values or by combined statistical/fold changes values.

3. Results

3.1. WM/GM ratios as estimates of glial and neuronal origin of gene transcripts

Based on a 50% cut-off value (WM/GM > 1.5 or <−1.5), the proportions of genes delineated by WM/GM ratios in the human and mouse brains are described in Table 2. A considerable overlap in gene selection was observed across datasets and was assessed by Pearson correlation of WM/GM ratios over all genes. WM/GM ratios were highly conserved across brain areas, cohorts and species, yielding significant correlated graphs (mean correlation = 0.63 ± 0.20 S.D., Fig. 1A). Highest correlations were observed between two different PFC areas within the same cohort ($R > 0.9$), and lower correlations between human cortical areas and mouse DG ($R \sim 0.36$, Fig. 1B).

WM/GM ratios for selected neuronal (CACNA/B, CALB1, GABRA/B, GRIN2A, SYN2, CAMK2A, etc.) and glial markers (AQP1, GFAP, CNP, EDG2, GSN, MBP, MOBP, MOG, OLIG2, PMP22) widely confirmed the validity of WM/GM ratios to estimate neuronal/glial origin of transcript (Table 3 and Supplementary Tables). We performed an additional literature survey for 40 genes with high or low WM/GM ratios across all three cohorts and confirmed in all cases that WM/GM ratios accurately predicted the expression of genes in neurons or glial cellular populations. Accordingly, genes similarly expressed

Table 2
Proportion (%) of genes defined by WM/GM ratios in human and mouse brains

	WM/GM<-1.5 ~neuronal-enriched	−1.5 <WM/GM < 1.5 ~expressed in both	WM/GM > 1.5 ~glial-enriched
Human			
PFC-*BA9*	27.7	46.7	25.6
PFC-*BA47*	26.9	47.6	25.5
ACG	32.5	40.2	27.3
AMY	13.4	66.8	19.8
Mouse			
FC	20.1	60.4	19.5
AMY	20.4	60.5	19.1
DG	22.8	58.2	19

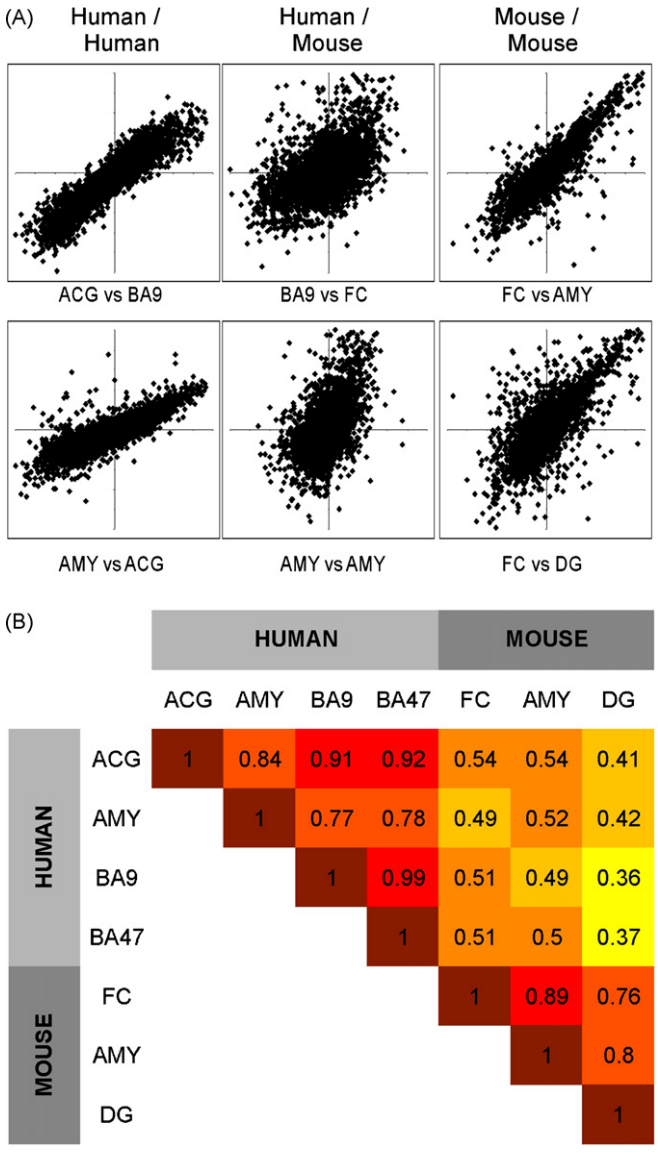

Fig. 1. Brain area and cross-species correlations of WM/GM ratios. (A) Correlation graphs of WM/GM ratios for human/human (~17,000 genes), human/mouse (~6100 orthologous genes) and mouse/mouse (~21,000 genes) brain area comparisons. Axis scales, $-6 < \log_2(\text{WM/GM}) < 6$. See also Fig. 3A. (B) Pearson correlation factors for area and species comparisons of WM/GM ratios. Significance of correlations, $p < e^{-7}$ for all r.

in both cell populations displayed ratios close to one, while low WM/GM ratio reflected neuronal enrichment, due to low WM signals. Within GM samples, a mean (±S.D.) ~22 ± 4% of detectable gene transcripts displayed neuronal-enrichment and ~23 ± 6% revealed glial-enrichment, whereas ~55 ± 9% of genes were similarly expressed in both cellular populations. It should be noted that genes display a gradient of enrichment in expression and that the 50% threshold of enrichment applied here can easily be customized based on the desired degree of stringency for any particular analysis (see Section 4). For instance, GFAP ratios vary from 1.4 to 1.9 in human brain areas (Table 3). Accordingly, as ratios are continuously distributed, lower (30–40%) or higher (60–80%) enrichment cut-offs will shift genes from the category of enriched genes to the pool of genes "expressed in both cell populations".

3.2. Selected applications of WM/GM ratios to transcriptome analysis of brain function

Combining *a priori* knowledge about cellular origin of transcripts into exploratory investigation of transcriptomes proved useful at numerous levels, mostly by providing new analytical perspectives that were otherwise not readily available. The cases provided here were based on our own studies, but the approach can be extended to any experiment in which restricting the pool of investigated genes based on information about cell-type expression can augment the analytical specificity.

3.2.1. Gene-wise information and glial/neuronal cellular programs

Literature surveys associate specific glial or neuronal functions to a small proportion of genes, corresponding mostly to cellular markers. Here, WM/GM ratios identified relative cellular origins for 1000 s of genes (Table 2), providing greatly increased knowledge about glial/neuronal cellular programs acquired in the anatomical context of the experiment at hand.

3.2.2. Neurons support area-specific gene expression

Differences in gene expression between brain areas likely reflect different cellular composition and functional specificities. For instance, for genes displaying brain area differences in transcript level at the $p < 0.01$ statistical level (two group t-test comparisons), ~1200 genes were expressed at a 50% higher level in human ACG *versus* AMY, while ~1600 genes displayed 50% higher levels in AMY *versus* ACG in cohort 2. Out of these groups of genes displaying area differences in expression levels, only 9.9% were glial-enriched in ACG, while 40.8% of the ~1600 AMY genes were glial-enriched.

In contrast, using a threshold of fivefold-enrichment in order to identify genes that are more "area-specific", over 90% of the 47 "ACG-specific" and of the 36 "AMY-specific" genes displayed WM/GM ratios that were indicative of neuronal origin of expression. Similar results were observed in the mouse cohort, suggesting that "area-enrichment" may reflect the structural difference in WM or glial contents of each brain area, while "area-specificity" appears to be supported by neuronal functions.

3.2.3. Assessing WM content across samples

Although WM/GM ratios represent average values across numerous samples, variations in overall glial transcript levels may be expected between GM samples. The source of this variability may be due either to biological differences in glial content within GM across subjects, or to the sampling protocol as it can be technically difficult to completely avoid WM contamination when collecting GM samples. We addressed this question by measuring the average deviation in transcript levels for the 100 most glial-enriched genes for each individual sample, when compared to the averaged transcript level for the same genes at the group level. This approach assumes that if a particular sample has overall more or less glial content, then all enriched

Table 3
WM/GM ratios for selected glial and neuronal markers

Gene title	Gene symbol	Human brain				Mouse brain		
		ACG	AMY	FC-BA9	FC-BA47	FC	AMY	DG
Calcium/calmodulin-dependent protein kinase II alpha	CAMK2A	−5.4	−2.4	−73.2	−50	−4.4	−3.6	−6.4
Neurogenic differentiation 1	NEUROD1	−5.8	−6.5	−49.7	−50.9	−5.8	−4.8	−21.8
Potassium channel, subfamily V, member 1	KCNV1	−22.9	−6.6	−41.2	−39.0	−6.3	−2.0	−2.5
Calbindin 1, 28 kDa	CALB1	−16.3	−2.9	−12.0	−12.3	−2.4	−6.3	−9.3
Gamma-aminobutyric acid (GABA) B receptor, 2	GABBR2	−12.9	−2.7	−14.6	−12.7	−5.1	−5.0	−5.1
Glutamate receptor, ionotropic, *N*-methyl D-aspartate 2A	GRIN2A	−8.8	−3.1	−5.3	−4.8	−6.5	−4.5	−22.0
Calcium channel, voltage-dependent, beta 2 subunit	CACNB2	−21.0	−4.8	−8.0	−7.2	−3.2	−3.5	−4.0
Cannabinoid receptor 1 (brain)	CNR1	−10.4	−2.9	−3.3	−2.8	−6.3	−7.4	−5.9
Gamma-aminobutyric acid (GABA) A receptor, alpha 1	GABRA1	−8.3	−1.9	−8.3	−8.0	−5.6	−3.4	−2.7
Synapsin II	SYN2	−9.8	−3.6	−4.4	−4.6	−1.6	−4.3	−4.4
Calcium channel, voltage-dependent, beta 3 subunit	CACNB3	−8.3	−3.2	−5.7	−5.6	−2.6	−2.5	−2.2
Gamma-aminobutyric acid (GABA) A receptor, alpha 2	GABRA2	−5.8	−2.6	−5.9	−5.6	−1.5	−3.3	−3.8
Calcium channel, voltage-dependent, alpha 1B subunit	CACNA1B	−10.9	−3.2	−2.1	−1.9	−1.7	−2.7	−3.6
Calcium channel, voltage-dependent, alpha 1G subunit	CACNA1G	−5.8	−1.8	−4.3	−4.1	−2.5	−2.7	−2.8
Glial fibrillary acidic protein	GFAP	1.9	1.4	1.9	1.8	8.0	9.6	3.0
Oligodendrocyte lineage transcription factor 2	OLIG2	6.2	2.8	4.8	5.0	4.1	3.7	5.0
Peripheral myelin protein 22	PMP22	6.1	2.3	4.2	4.6	3.7	6.5	4.5
Myelin basic protein	MBP	8.2	2.4	5.6	5.5	3.6	4.2	3.8
2′,3′-Cyclic nucleotide 3’ phosphodiesterase	CNP	5.3	2.3	3.5	3.6	9.7	10.4	11.5
Gelsolin	GSN	6.3	4.3	2.8	2.9	8.8	12.1	12.1
Myelin oligodendrocyte glycoprotein	MOG	12.0	3.6	3.9	4.4	13.9	19.6	15.9
Myelin-associated oligodendrocyte basic protein	MOBP	6.1	3.3	5.5	5.2	15.3	23.2	19.2
Aquaporin 1	AQP1	4.0	4.9	2.7	2.4	26.5	26.5	26.4
Endothelial differentiation, lysophosphatidic acid GPCR, 2	EDG2	6.6	2.4	4.5	4.6	24.7	28.8	32.5

Values were converted in WM/GM fold changes. See Tables S1–3 for all genes on respective arrays.

transcripts should be similarly affected. Compared to mean values within each cohort, mean (±S.D.) sample deviations in WM were 9.8 ± 4.8% in BA9 and 9.1 ± 5.4% in BA47 in cohort 1, 16.2 ± 7.8% in ACG and 12.0 ± 5.8% in AMY in cohort 2, and from 6.3 ± 4.4% in FC, 7.7 ± 6.2% in AMY and 10.2 ± 6.1% in DG in the mouse cohort. The presence of samples from two closely related PFC areas in cohort 1 allowed us to further assess the source of this variability. Indeed, deviations from average WM content were significantly correlated between BA9 and BA47 samples within subjects ($r = 0.51$ and $p = 0.002$) (Fig. 2) and larger discrepancies were observed only for a minority of samples (black circles in Fig. 2), potentially reflecting collection artifacts for these few samples. Accordingly, the overall conservation of WM content across related brain areas within subjects suggested a biological origin of GM glial content.

Since relative WM contents can be estimated for individual GM samples, one can also compare average contents across experimental groups and estimate deviation in glial content according to clinical diagnosis for instance. As different pathologies may occur in neuronal and glial cell population in psychiatric disorders, we have measured deviations in WM content in controls and depressed suicide victims in cohort 1. In that instance, no differences were observed ($p > 0.1$, see Sibille et al., 2004), suggesting similar glial content across experimental groups. This approach does not test the presence of changes within specific glial subtypes, but in that case ruled out an overall change in glial/neuronal ratio within GM samples, as estimated by WM/GM ratios.

3.2.4. Altered neuron/glia ratios versus cell-type specific changes?

Contrary to overall deviation in WM content, a more restricted biological event would essentially leave most high-

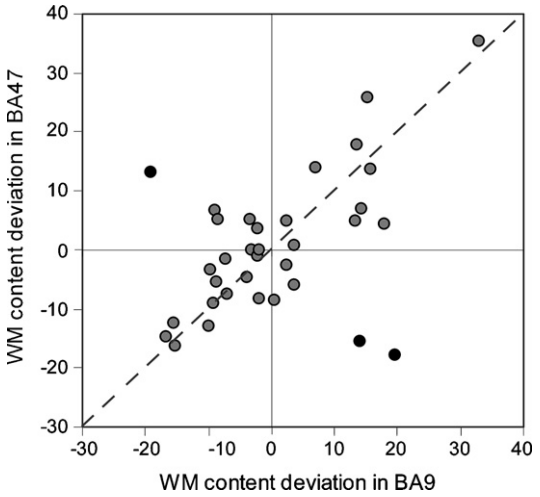

Fig. 2. The WM/glial content of GM samples is mostly subject-dependent. Dots represent the average percentile of deviation in WM content per sample across the two PFC areas in cohort 1. Most samples displayed variable levels of WM content within GM samples, although values agreed well across two closely related PFC areas, suggesting a biological determination of glial content within subjects. Three GM samples display larger discrepancies in their WM content across areas (black dots) ($r = 0.51$ and $p = 0.002$ for all samples; $r = 0.83$ and $p < e^{-7}$ for gray samples). Hashed bars represent line of similar values (slope = 1).

enriched genes unchanged. This notion is illustrated here in the context of our previous characterization of molecular aging in PFC in cohort 1 (Erraji-BenChekroun et al., 2005). In this cohort spanning seven decades of life, 588 genes were identified as differentially expressed with age. Fig. 3 shows the high correlation of WM/GM ratios between two closely related regions of the PFC (BA9 and BA47; $R = 0.99$ from Table 1). Age-affected genes were superimposed on that graph (red dots in Fig. 3A). The continuous distribution of age-affected genes clearly demonstrated that all levels of glial/neuronal enrichment

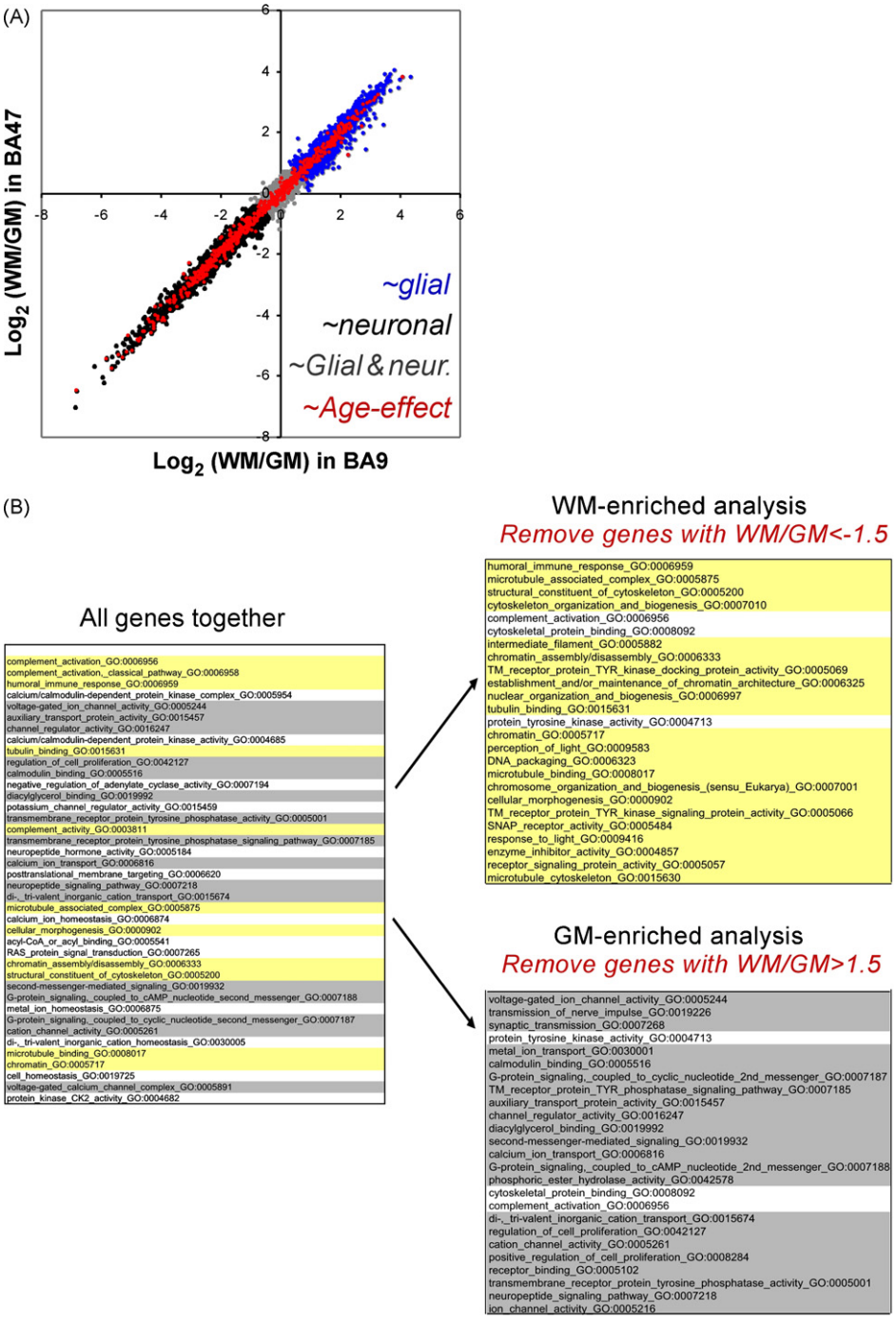

Fig. 3. Using WM/GM ratios highlights the extent and cell-type specificity of age-related transcriptome changes. (A) Age-effected genes superimposed on WM/GM correlation graph in human PFC. Age-affected genes (red) represented only 14% of the most neuronal genes (black) and 6% of the glial (Blue) genes and were evenly distributed across the spectrum of WM/GM ratios. (B) Functional analysis based on WM/GM transcript enrichment separates the contribution of glia and neurons to molecular aging. (Left) "All genes combined" gene ontology (GO) analysis of altered gene expression in aging PFC. The top 40 most affected gene groups are presented out of ~900 groups investigated. (Right) Analysis of the same dataset using WM- or GM-enriched gene pools identified two distinct sets of functions. Altered glial-related gene groups (yellow, upper right) mostly concerned cellular defenses, whereas most age-affected neuronal gene groups (gray, lower right) related to synaptic functions and signal transduction. The top 25 most affected gene groups are presented for each analysis. White bars indicated functions affected in both cellular populations. Details in Erraji-BenChekroun et al. (2005).

were represented and also showed that the tails of the distribution were mostly unaffected by age. Thus, a simple visual check of the graph quickly assessed the extent of the effect of aging, and demonstrated that aging correlated with selective cellular and molecular changes, rather than an overall change in the neuron/glia ratio within GM (Erraji-BenChekroun et al., 2005).

3.2.5. *WM/GM-based functional analysis of transcriptomes segregates glial and neuronal biological effects*

To identify affected biological functions between two experimental states, differences in transcript level are frequently investigated within biological pathways or groups of genes with related functions (Dennis et al., 2003; Pavlidis et al., 2002). This is typically performed by investigating over-representation of affected genes within reference groups that correspond to specific biological, cellular or molecular functions. Thus, restricting the number of genes included in the reference groups increases the specificity of the analysis by limiting the diluting effect of genes with unrelated effects.

With regards to aging, analyzing all genes together yielded a list of biological functions potentially affected during aging (Fig. 3B left). However, restricting the pool of investigated genes to glial- or neuronal-enriched gene sets readily segregated results in putative glial- and neuronal-related events (Fig. 3B). Segregated results also showed greater consistency, as most glial functions related to cellular defenses and inflammation, while neuronal functions affected by age corresponded mostly to synaptic and signal transduction. Moreover, the presence of altered transcript levels for known cellular markers within specific functional groups (i.e., GFAP and inflammation) helped formulate the hypothesis of a more restricted astrocyte phenotype during brain aging (see detailed analysis and discussion in Erraji-BenChekroun et al., 2005). These findings were in very good agreement with current knowledge about mechanisms of aging, thus representing a biological validation of the WM/GM "add-on" analysis, as a rapid first step to identify potential contributions of neurons and glia to altered transcripts and functions within combined GM samples.

4. Discussion

In summary, we have described a simple approach that uses estimates of relative neuronal/glial origin of transcripts to greatly enhance transcriptome analyses of complex GM brain samples. We show that incorporating WM/GM ratios into transcriptome analysis (i) provides new and different analytical perspectives otherwise not available, (ii) increases the potential for biological insight obtained from postmortem transcriptome studies, (iii) expands knowledge about glial and neuronal cellular programs and (iv) facilitates the generation of cell-type specific hypotheses. The main feature of the WM/GM differential analytical approach resides in its robustness since ratios are highly conserved across areas and species, and results rely on converging effects on large numbers of genes. Moreover, as WM/GM ratios are continuously distributed, analytical thresholds can easily be adjusted based on the extent of the primary biological effect investigated, and on the desired degree of exploratory potential. The second main feature is its versatility, as this approach can be combined with any analytical approach where cell-type specific information is relevant.

What do WM/GM ratios represent? These ratios were generated by comparing transcript levels in GM and adjacent WM samples. These two tissues have different cellular compositions. Specifically, glial cells are widely distributed throughout WM and GM, while neuronal populations are found predominately in GM. Our results suggest that WM/GM ratios provide reliable estimates of glial or neuronal origin of transcripts. Ratios for oligodendrocyte-specific genes for instance consistently provided WM/GM ratios that indicated a higher presence in WM, in agreement with their expected over-representation in WM tracts (Table 3). Astrocytes, on the other hand, are more widely distributed across WM and GM, yet astrocyte markers (GFAP) still produced WM/GM ratios > 1.5 indicative of a glial origin of expression. It is important to note that this approach is also not expected to work for all individual genes in most brain areas or under different conditions, as glial cells may differentially recruit various cellular programs depending on their environment and functional levels. In other words, our analysis demonstrated the sensitivity of the approach, but selectivity may vary for some individual genes, depending on the functional and genetic specialization of the brain areas investigated. Nevertheless, overall WM/GM ratios were remarkably conserved (Fig. 1), despite expected brain region or species differences in WM content. This was also notable considering that datasets had been collected at different times, in separate cohorts, in different species, and despite differences in cellular compositions of the respective GM areas and the much reduced amount of WM in mouse brain compared to the human brain. Accordingly, the observed variations in group proportions (Table 2) and correlations (Fig. 1B) within these conserved boundaries likely reflected the anatomical characteristics and distribution of WM tracts across brain regions and species. For instance the lower proportion of WM- and GM-enriched genes in rodent samples may correspond to the overall reduced WM content in rodent brain and the scarcity of pure WM areas that are available for dissection in the mouse brain. Similarly, the increased proportion of genes with WM/GM ratios in the range of −1.5 to +1.5 may reflect the more diffuse presence of WM tracts within that brain areas, compared to cortical gray matter.

The conservation of WM/GM ratios over large group of genes under various circumstances underscores the major strengths of the approach, which are its robustness and versatility. Indeed, WM/GM ratios are continuously distributed and thresholds of enrichment can easily be adjusted, using either empirical or statistical values. Here, we have used an arbitrary cut-off value of 50% for our analyses, which separated genes in three pools: ∼22% neuronal-enriched, ∼23% glial-enriched and ∼55% expressed in both cellular populations. Incorporating this information into analysis of biological events provided interesting new perspectives that were otherwise not available, including neuronal support of regional differences, delineation of age effect (specificity *versus* overall glial effect), distinct glial and neuronal functional correlates of aging, assessment of WM content across samples, and deviation in WM/glial content

according to clinical diagnosis. As distinct neuronal and glial pathologies have been formulated for most psychiatric disorders (Cotter et al., 2001; Hakak et al., 2001; Hamidi et al., 2004; Rajkowska et al., 1999), it is important to develop tools that can assess the degree of specificity of glial-related findings. Here, we have presented data on WM/GM ratios generated in control subjects only, however results using all samples were identical ($WM/GM_{controls}$ *versus* $WM/GM_{all\ samples}$, Pearson correlations ~0.98–1.0), thus ruling out any correlation between psychiatric disorders and altered glia/neuron ratios in cortical GM samples in cohort 1 (Sibille et al., 2004).

The analyses presented here typically rely on large number of genes and have yielded similar results using different analytical thresholds. Accordingly, we have not provided strict statistical guidelines on purpose, but rather we have emphasized the exploratory potential of applying WM/GM ratios to various transcriptome analyses. It is our experience that the final balance between analytical thresholds and results is determined by the strength of the biological event investigated and the degree of exploratory analysis that is desired. Here, we have used published knowledge about glial and neuronal expression, as well as about aging, to validate some of the applications of WM/GM ratios. The gene expression correlates of aging are widespread, robust and well characterized (Erraji-BenChekroun et al., 2005; Lee et al., 2000; Lu et al., 2004) and thus offer ideal internal controls for validating exploratory analyses. Indeed, similar results were consistently obtained with variable cut-offs and survived more stringent analytical conditions (Erraji-BenChekroun et al., 2005). Importantly, we view the use of WM/GM ratios as an "add-on" feature that facilitates rapid sorting of genomic results, while supporting hypothesis generation. In particular, it offers a wider view of overall patterns relating to glial and neuronal functions that are otherwise not accessible when simply looking at individual differences in transcript levels in the group of genes. The example of the differential functional analysis of molecular aging, combined with the presence of cellular markers of astrocytes within identified functions, clearly demonstrated the increased specificity of WM/GM-assisted analysis, and highlighted the power of this approach in helping establish hypotheses for cell-type specific biological events or molecular pathologies (e.g., astrocyte phenotype in aging; details in Erraji-BenChekroun et al., 2005).

Importantly, this approach does not replace systematic analyses of WM samples as potential primary sites of pathology, nor does it offer the specificity of laser-captured cell types. As mentioned before, it is also not expected to identify all genes within glial or neuronal cellular programs, as functional status, composition of the brain area investigated, or dissection protocols may affect transcript levels and sensitivity to detect differences. Also, while WM/GM ratios performed well for estimation of relative glial- and neuronal-origin of transcripts, they do not replace the confirmation of cellular expression by anatomical approaches, such as dual-label in situ hybridization or immunohistochemistry. What our results suggest is that combining a WM/GM differential analysis with traditional investigation of transcriptomes provides added perspectives on glial or neuronal events that are otherwise not available using traditional approaches. Furthermore, we have shown that investigating cellular markers in combination with WM- of GM-based functional analysis can synergize both approaches and lead to cell-type-specific hypotheses.

Additional limitations include that samples from cohorts 1 and 2 were generated at different times, used different brain banks, and focused on different brain areas. Directly comparing samples from similar areas would have been useful, however a strength of the study is that WM/GM ratios were conserved across areas, despite these limitations. This observation may not be so surprising considering that glia are potentially 10 times more numerous than neurons in the brain. Specifically, since glial populations are predominant and less diverse than neuronal population, averaged gene-wise WM values are expected to be robust and constant between samples and to vary little across brain areas. These expected stable WM values potentially compensate for more variable GM values, which include transcript values from both types of cells.

As described in the text, most cellular enrichment of candidate genes was confirmed; however ~10–15% of genes surveyed displayed ratios that were close to, or below the enrichment threshold used in this report. For instance, GFAP ratios vary from 1.4 to 1.9 in human brain areas (Table 3). Moreover, all multiple probesets for the same gene did not necessarily agree, as variable efficiencies and alternate gene structure may affect signals for a same gene across probesets. In view of these limitations, it is important to note that since ratios are continuously distributed, lower (30–40%) or higher (60–80%) enrichment cut-offs will shift numerous genes from the category of enriched genes to the pool of genes "expressed in both cell populations". Furthermore, adjusting the initial selection of genes may also affect results. Here we have removed genes with expression level below 50. Including transcripts with lower abundance are expected to progressively yield blunted or less accurate ratios, as detection of gene transcript levels approach background levels. Together, adjustments need to be made on a case-by-case basis, depending on the type of analysis performed and stringency desired.

Finally, as high correlations between cohorts, array types, and related brain areas highlighted the robustness of WM/GM ratios, they also suggested that WM/GM-based analyses could be applied *post hoc* to related datasets. To this end we have provided tables of WM/GM ratios generated in the described mouse and human brain areas (Tables S1–3) and included guidelines for use.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneumeth.2007.08.009.

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