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Comparative pathway and network analysis of brain transcriptome changes during adult aging and in Parkinson's disease

Enrico Glaab *, Reinhard Schneider

Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, Luxembourg

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ABSTRACT

Aging is considered as one of the main factors promoting the risk for Parkinson's disease (PD), and common mechanisms of dopamine neuron degeneration in aging and PD have been proposed in recent years. Here, we use a statistical meta-analysis of human brain transcriptomics data to investigate potential mechanistic relationships between adult brain aging and PD pathogenesis at the pathway and network level. The analyses identify statistically significant shared pathway and network alterations in aging and PD and an enrichment in PD-associated sequence variants from genome-wide association studies among the jointly deregulated genes. We find robust discriminative patterns for groups of functionally related genes with potential applications as combined risk biomarkers to detect aging- and PD-linked oxidative stress, e.g., a consistent over-expression of metallothioneins matching with findings in previous independent studies. Interestingly, analyzing the regulatory network and mouse knockout expression data for NR4A2, a transcription factor previously associated with rare mutations in PD and here found as the most significantly under-expressed gene in PD among the jointly altered genes, suggests that aging-related NR4A2 expression changes may increase PD risk via downstream effects similar to disease-linked mutations and to expression changes in sporadic PD. Overall, the analyses suggest mechanistic explanations for the age-dependence of PD risk and reveal significant and robust shared process alterations with potential applications in biomarker development for pre-symptomatic risk assessment or early stage diagnosis.

Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders and a disease-modifying therapy is still not available. With an average age of onset of 60 years and a risk of developing sporadic PD known to increase significantly with age, the disease has been linked with aging by several studies (Bender et al., 2006; Collier et al., 2011; Frey et al., 2004; Hindle, 2010; Levy, 2007; Kaasinen and Rinne, 2002; Naoi and Maruyama, 1999). Previous hypotheses have suggested a combination of age-related neuronal attrition and environmental factors as a major cause for sporadic PD (Calne et al., 1986) or that aging may influence the clinical progression of the disease (Levy, 2007). More recently, PD has also been proposed to represent a form of premature or accelerated aging (Collier et al., 2011). Independent of the type and extent of association between aging and PD pathogenesis, various shared molecular hallmarks have been observed, including a gradual decline in dopamine synthesis (Scatton et al., 1983; Ota et al., 2006), reduced striatal density of the type 2 vesicular monoamine transporter (Frey et al., 2004) and increased levels of deleted mitochondrial DNA (Bender et al., 2006). These common features suggest that a more comprehensive investigation of shared/interlinked cellular process changes in aging and PD could provide new insights on the disease etiology and progression and facilitate the discovery of pre-symptomatic risk biomarkers for PD or general neurodegeneration.

In recent years, large-scale transcriptomic measurements from research studies on brain aging and complex neurodegenerative disorders have been made available in public data repositories (Barrett et al., 2009; Kang et al., 2011; Jones et al., 2009; Lein et al., 2006). Although these data sources have been analyzed individually (Kang et al., 2011; Johnson et al., 2009; Zhang et al., 2005; Lesnick et al., 2007; Kumar et al., 2013), the potential for a joint pathway- and network-analysis of high-throughput gene expression data for aging and PD has not yet been exploited, despite PD being regarded as one of the prime examples of an age-related disease (Hindle, 2010).

Here, we investigate relations between brain transcriptome changes in PD patients (as compared to age-matched, non-demented control subjects) and transcriptome changes associated with adult brain aging in a separate group of unaffected individuals, exploiting new cross-study data integration, pathway and network analysis methods. Specifically, we first apply a recent statistical meta-analysis approach (Marot et al., 2009) to 8 public microarray gene expression data sets (Zhang et al., 2005; Lesnick et al., 2007; Moran et al., 2006; Simunovic et al., 2009; Zheng et al., 2010), using *post mortem* samples from the midbrain

* Corresponding author at: Luxembourg Centre for Systems Biomedicine, 7, avenue des Hauts-Fourneaux, L-4362, Esch-sur-Alzette, Luxembourg. Fax: +352 466644 6949.
E-mail address: enrico.glaab@uni.lu (E. Glaab).
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substantia nigra region in patients and age- and gender-matched controls, and then compare the differentially expressed genes in PD from the meta-analysis to genes associated with adult brain aging. These aging-associated genes are derived from a statistical analysis of *post mortem* microarray samples from the Human Brain Transcriptome (HBT) project (Kang et al., 2011), by determining significant brain gene expression changes across different age groups during adulthood. By integrating these data to identify shared and associated gene expression alterations during natural brain aging and in PD, we aim at two main goals: (1) obtaining a more detailed molecular-level understanding of how aging contributes to the risk for PD and (2) finding robust shared alterations in PD and aging for further evaluation as candidate early risk biomarkers for PD or general neurodegeneration. The potential of the jointly altered genes for biomarker applications is investigated using public data to determine their expression in peripheral tissues, to identify their previously reported PD/aging-linked peripheral changes and to evaluate the specificity of their alterations in PD as compared to Alzheimer's disease.

These analyses at the single-gene level are complemented by a statistical assessment of cellular processes changes, using our previously developed pathway and network analysis method EnrichNet (Glaab et al., 2012) to identify shared significant pathway and sub-network deregulations in PD and aging. The transcription factors most relevant for the regulation of these affected sub-networks are predicted using an over-representation analysis for transcription factor binding sites among the altered genes in PD/aging. Finally, to investigate possible relations between genetic variations and transcriptome alterations linked to PD/aging, we test the enrichment of PD-associated single-nucleotide sequence variants (SNPs) from public genome-wide association studies (GWAS) among the altered genes in PD/aging and report the genes found significant in both transcriptomics and GWAS analyses.

Materials and methods

Microarray data collection, pre-processing and differential expression analysis

In order to exploit the synergies of the available transcriptomics data for a joint analysis of PD pathogenesis and brain aging, we collected *post mortem* samples in the *substantia nigra* midbrain region from public PD case–control microarray data sets, as well as *post mortem* microarray samples from the Human Brain Transcriptome (HBT) project for 3 adult age periods (20 to 40 years, 40 to 60 years, and 60 years onwards; used to identify aging-associated changes in brain gene expression during adulthood, see below).

For the meta-analysis of *substantia nigra* brain samples from PD case–control studies, raw microarray data were obtained from 8 published data sets (Zhang et al., 2005; Lesnick et al., 2007; Moran et al., 2006; Simunovic et al., 2009; Zheng et al., 2010). Importantly, the samples were already age- and gender-matched to prevent biases in downstream analyses. All microarray data sets were pre-processed using the GC-RMA procedure for background correction, normalization and probe replicate summarization (Wu et al., 2004), and only samples from the *substantia nigra* brain region were retained for further analysis. Since platform-specific biases can lead to artifacts when directly integrating microarray data from different studies via cross-study normalization methods, we instead used a meta-analysis to integrate statistical results obtained on the individual data sets. First, differential expression statistics were computed on each data set separately using the empirical Bayes moderated *t*-statistic (Smyth, 2004), and then the *p*-value significance scores were combined via the weighted meta-analysis approach by Marot et al. (2009). In contrast to the commonly used unweighted Fisher method for *p*-value combination, this approach involves data set-specific weights reflecting the relative number of samples collected in each study. The obtained meta-analysis *p*-values were adjusted for multiple hypothesis testing using the approach by Benjamini and Hochberg (1995) and a false discovery rate (FDR) threshold of 0.05 to determine the final gene selection. Since two of the microarray data sets were derived using laser-capture microdissection (LCM), we confirmed the consistency between LCM and non-LCM data by determining the Spearman correlation between the median fold changes across the LCM- and the non-LCM data sets for the genes considered in this study (Spearman's rho = 0.634) and the significance of the linear regression fit between these two data series ($p = 2.19E-09$).

For the aging-related microarray data from the Human Brain Transcriptome project (Kang et al., 2011) (HBT), all samples covering the 3 main adult age periods 20 to 40 years, 40 to 60 years and 60 years onwards were collected in order to identify differentially expressed genes across these age groups. Importantly, as mentioned in the original publication for the HBT project, none of the individuals included in this study suffered from any known neurological or psychiatric disorder, severe head injuries or signs of neurodegeneration (Kang et al., 2011). The significance of differential expression across the age groups was computed using a dedicated multiclass-analysis method designed for microarray data (Tusher et al., 2001). We chose this specific approach in order to identify increases or decreases in gene expression variance related to aging in addition to positive or negative correlations with aging (the correlation with aging is additionally reported in Tables 1 and 2, and for the full-length gene ranking table in the Supplementary Material). Finally, the heat map visualizations in Figs. 1, 2 and 3 were generated using a Pearson correlation hierarchical clustering (i.e., the distance metric is 1-correlation; larger versions of these heat maps including the gene names, as well as heat maps for a Euclidean distance metric and additional sample clustering are provided in the Supplementary Material, see Fig. S1–S8).

Network-based enrichment analysis of PD and aging transcriptomics data

To analyze associations between the deregulated genes in PD/aging and cellular pathways and exploit additional information from public molecular interaction data, we used our algorithm *EnrichNet* for network-based gene/protein set enrichment analysis (see Glaab et al., 2012 for a detailed description and the publicly available web-application www.enrichnet.org). Briefly, EnrichNet consists of a 3-step procedure: First, a gene or protein set of interest (the target gene set) as well as gene/protein sets representing cellular pathways from public databases (the reference gene sets) are mapped onto a genome-scale protein–protein interaction network. Next, a deterministic procedure for simulating random walks in a network (the Random Walk with Restart algorithm Tong et al., 2008) is applied to score the network distances and multiplicity of interactions between the target and reference gene/protein sets. In order to obtain final association scores for the pathway reference sets, the combined interconnectivity/distance scores are compared to a background score distribution using the XD-statistic (Olmea et al., 1999; Glaab et al., 2012) (larger XD-scores reflect stronger associations, and the algorithm determines an XD-score significance threshold corresponding to a false-discovery rate of 0.05).

Here, in order to identify and score network associations of the deregulated genes in aging and PD with known cellular pathways, we applied EnrichNet on a target gene set given by the intersection of the significant genes from the differential expression analyses of the aging and PD transcriptome data (FDR < 0.05, see above). The pathway-representing reference gene sets were obtained from the public databases Gene Ontology (Ashburner et al., 2000), KEGG (Kanehisa and Goto, 2000), WikiPathways (Pico et al., 2008) and Reactome (Joshi-Tope et al., 2005). To assemble the genome-scale protein–protein interaction network only experimentally verified, direct physical interactions from public data repositories including tissue-specificity annotations (Bossi and Lehner, nd.) were used. In addition to the network association scores obtained from the graph-based statistic, we also performed a conventional over-representation analysis, scoring the significance of the overlap between target and

Table 1
Significantly differentially expressed genes in both Parkinson's disease and adult brain aging (Top 50, FDR < 0.05, see Supplementary Material for the complete list).

Gene symbol (HGNC)	Gene description	Deregulation in PD samples (Z–score)	Correlation with aging
NR4A2	nuclear receptor subfamily4, group A, member 2	−7.43	−0.53
NAP1L2	nucleosome assembly protein 1–like 2	−7.13	−0.31
PEG10	paternally expressed 10	−7.06	−0.53
MCM7	minichromosome maintenance complex component 7	6.86	0.2
CLK1	CDC–like kinase 1	6.32	0.42
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	−6.3	−0.39
STAM	signal transducing adaptor molecule 1	−6.28	−0.57
KCNJ2	potassium inwardly–rectifying channel, subfamily J, member2	6.16	−0.39
SLIT1	slithomolog1 (Drosophila)	−6.13	−0.43
CDH8	cadherin 8, type 2	−6.09	−0.54
GRIA1	glutamate receptor, ionotropic, AMPA1	−6.06	−0.51
SERINC3	serinein corporator 3	−6.05	−0.47
KAZ	kazrin	6.01	−0.54
MAP3K9	mitogen–activated protein kinase kinase kinase 9	−5.97	−0.41
MYT1L	myelin transcription factor 1–like	−5.8	−0.58
C12orf43	chromosome 12 open reading frame 43	−5.51	−0.35
PLD3	phospholipase D family, member 3	−5.4	−0.2
BSN	bassoon (presynaptic cytomatrix protein)	−5.46	−0.3
MORC2	MORC family CW–type zinc finger 2	5.43	−0.41
SUN2	Sad 1 and UNC 84 domain containing 2	5.39	−0.28
CACNA1G	voltage–dependent calcium channel, Ttype, alpha 1G subunit	−5.3	−0.6
ATP6V1F	ATPase, H+ transporting, lysosomal 14kDa, V1subunitF	−5.14	0.32
NHLH2	nescient helix loop helix 2	−5.03	0.26
FAM49A	family with sequence similarity 49, member A	−4.99	−0.45
AAK1	AP2 associated kinase 1	−4.93	−0.34
LARP1	La ribonucleoprotein domain family, member 1	−4.92	−0.5
ZNF365	zinc finger protein 365	−4.86	0.48
CREBBP	CREB binding protein	4.68	−0.48
ARHGEF10	Rho guanine nucleotide exchange factor (GEF) 10	4.57	−0.25
SDCCAG3	serologically defined colon cancer antigen 3	4.55	0.34
NDUFB2	NADH dehydrogenase (ubiquinone) 1beta subcomplex, 2	−4.51	0.38
MT1H	metallothionein 1H	4.42	0.27
AFF2	AF4/FMR2 family, member 2	−4.37	−0.58
CCDC92	coiled–coil domain containing 92	−4.36	−0.54
CDKN1A	cyclin–dependent kinase inhibitor 1A (p21, Cip1)	4.33	0.38
MT1G	metallothionein 1G	4.31	0.57
PHF10	PHD finger protein 10	4.29	0.4
FKBP11	FK506 binding protein 11, 19 kDa	−4.22	−0.62
CAMTA1	calmodulin binding transcription activator 1	−4.2	−0.44
ACSL1	acyl–CoA synthetase long–chain family member 1	4.16	−0.58
DUSP7	dual specificity phosphatase 7	4.15	−0.52
INF2	inverted form in, FH2 and WH2 domain containing	4.13	−0.37
ASCL1	achaete–scute complex homolog1 (Drosophila)	4.09	0.38
NPAS2	neuronal PAS domain protein 2	4.09	−0.42
PLEKHM1	pleckstrin homology domain containing, familyM member 1	4.05	−0.41
CALB1	calbindin 1,28kDa	−3.97	−0.59
PHB	prohibitin	3.95	0.01
LMF1	lipase maturation factor 1	−3.95	−0.47
BTBD3	BTB (POZ) domain containing 3	−3.93	−0.33
CLIC2	chloride intracellular channel 2	3.92	0.5

reference gene sets using Fisher's exact test with multiple testing corrections (Benjamini and Hochberg, 1995, both scores are provided in Table 3). Importantly, the network association score and the over-representation score may reflect different types of functional associations; hence, for both types of approaches, we report the identified pathways with estimated false-discovery rate below 0.05 (see Table 3 and Supplementary Material).

SNP over-representation analysis

To identify genes with PD-associated SNPs from public genome-wide association studies (GWAS) coinciding with the significantly altered genes in PD and aging obtained from the analyses described above, we collected SNPs with phenotype label “Parkinson's disease” from the Ensembl Variation database (Chen et al., 2010). These SNPs

Table 2
Molecular functions of deregulated genes in aging and PD.

Function	Gene symbol	Gene description	Deregulation in PD samples (Z–score)	Correlation with aging
Mitochondrial	NDUFB2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa	–4.51	0.38
	PHB	prohibitin	3.95	0.01
	ELAVL1	embryonic lethal, abnormal vision, Drosophila–like 1	3.01	–0.58
	PCK1	phosphoenolpyruvate carboxykinase 1 (soluble)	2.72	0.58
	HSD17B14	hydroxysteroid (17–beta) dehydrogenase 14	2.57	0.61
Neuron differentiation	CEBPB	CCAAT/enhancer binding protein(C/EBP), beta	3.6	0.39
	EPHB2	EPH receptor B2	–3.55	–0.49
	RASGRF1	Ras protein–specific guanin enucleotide–releasing factor 1	2.92	0.19
	ASCL1	achaete–scute complex homolog1 (Drosophila)	4.09	0.38
	CXCL12	chemokine (C–X–Cmotif) ligand 12	–2.63	–0.35
	CNTNAP2	contactin associated protein–like 2	–2.8	–0.33
	EFNB3	ephrin–B3	–3.43	–0.38
	NR4A2	nuclear receptor subfamily 4, groupA, member 2	–7.43	–0.53
	SLIT1	slit homolog1 (Drosophila)	–6.13	–0.43
Apoptosis	RASGRF1	Ras protein–specific guanine nucleotide–releasing factor 1	2.92	0.19
	EIF2AK2	eukaryotic translation initiation factor 2–alpha kinase 2	–3.92	–0.49
	GGCT	gamma–glutamylcyclotransferase	–3.12	–0.28
	PEG10	paternally expressed 10	–7.06	–0.53
	SRGN	serglycin	3.32	0.53
	TNFRSF21	tumor necrosis factor receptor superfamily, member 21	–6.3	–0.39
Cognition (learning, memory)	AFF2	AF4/FMR2 family, member 2	–4.37	–0.58
	EPHB2	EPH receptor B2	–3.55	–0.49
	RASGRF1	Ras protein–specific guanine nucleotide–releasing factor 1	2.92	0.19
	CALB1	calbindin 1, 28kDa	–3.97	–0.59
	GRIA1	glutamate receptor, ionotropic, AMPA 1	–6.06	–0.51
Inflammatory response	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	3.6	0.39
	ADORA3	adenosine A3 receptor	2.68	0.35
	ATRN	attractin	–3.02	–0.53
	NR4A2	nuclear receptor subfamily 4, group A, member 2	–7.43	–0.53
	C3AR1	complement component 3a receptor 1	2.67	0.01
Ionchannel	CACNA1G	calciumchannel,voltage–dependent, Ttype,alpha1Gsubunit	–5.3	–0.6
	CACNG3	calciumchannel,voltage–dependent,gammasubunit3	–3.17	–0.43
	KCNJ2	potassiuminwardly–rectifyingchannel, subfamily J, member 2	6.16	–0.39
	KCNB1	potassium voltage–gated channel Shab–related subfamily, member 1	–3.69	–0.41
	CLIC2	chloride intracellular channel 2	3.92	0.5
	GRIA1	glutamate receptor, ionotropic, AMPA 1	–6.06	–0.51
	P2RX5	purinergic receptor P2X, ligand–gated ion channel, 5	–3.49	–0.28
Lysosomal	NPC1	Niemann–Pick disease, type C1	3.01	–0.27
	SRGN	serglycin	3.32	0.53
	SMPD1	sphingomyelin phosphodiesterase1, acid lysosomal	–2.97	–0.37
Metalion homeostasis	MT1G	metallothionein 1G	4.31	0.57
	MT1H	metallothionein 1H	4.42	0.27
	AKR1C3	aldo–ketoreductase family1, member C3	3.49	0.45

were assigned to two groups depending on their *p*-value significance score: (1) SNPs with genome-wide significance (defined as $p < 10E\text{-}08$) and (2) suggestive SNPs (defined as $p < 0.001$). The over-representation of genes containing SNPs from group 1 and 2 among the jointly deregulated genes in aging and PD were both scored using the one-tailed Fisher exact test.

Results and discussion

Shared significant deregulations of individual genes in Parkinson's disease and brain aging

When determining the intersection between the differentially expressed transcripts/genes obtained from the PD meta-analysis with those from the aging data analysis, 120 significant transcripts (FDR < 0.05) mapping to unique shared genes were identified (the top 50 sorted by absolute Z-score across the 8 PD microarray data sets are listed in Table 1; the complete list is provided in the Supplementary Material). Heat map visualizations showing the expression levels of the 120 genes in PD samples vs. controls and in different age groups for unaffected individuals are displayed in Figs. 1 and 2 (Pearson correlation hierarchical clustering was applied, see Supplementary Material for alternative clustering approaches).

Apart from these gene-level clustering, an additional hierarchical clustering was applied to the samples (see Fig. 3) to identify potential grouping patterns among them and see whether the top-level clusters show an association with the disease and control sample groups. However, no such relation was identified, which may be explained by three possible reasons: (1) biological grouping patterns among the samples exist which are unrelated to the disease/control-group differences of

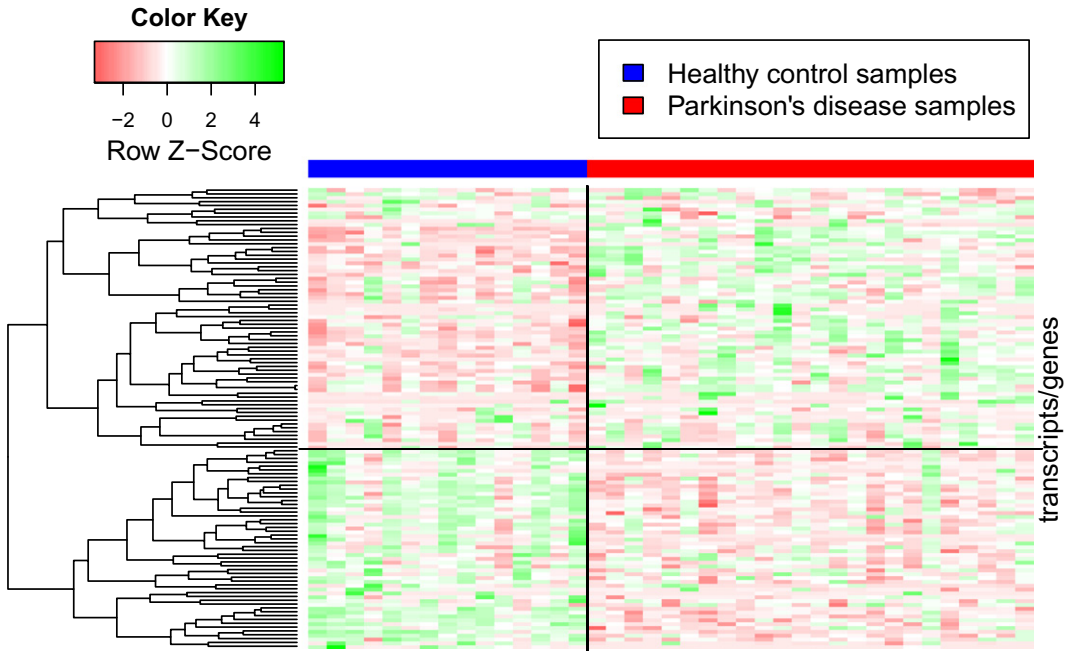

Fig. 1. Heat map for the expression levels of the 120 transcripts significantly altered in aging and PD in the data set by Moran et al. (2006), comparing brain samples from the *substantia nigra* in Parkinson's patients (red) against controls (blue; see also the heat map in Fig. 2 showing the same transcripts across different age groups). The plot uses a Pearson correlation hierarchical clustering for the genes (see dendrogram on the left and black horizontal line, indicating the top-level cluster separation). A larger version of this map and an alternative clustering with the Euclidean distance metric for both genes and samples including all gene labels is provided in the Supplementary Material.

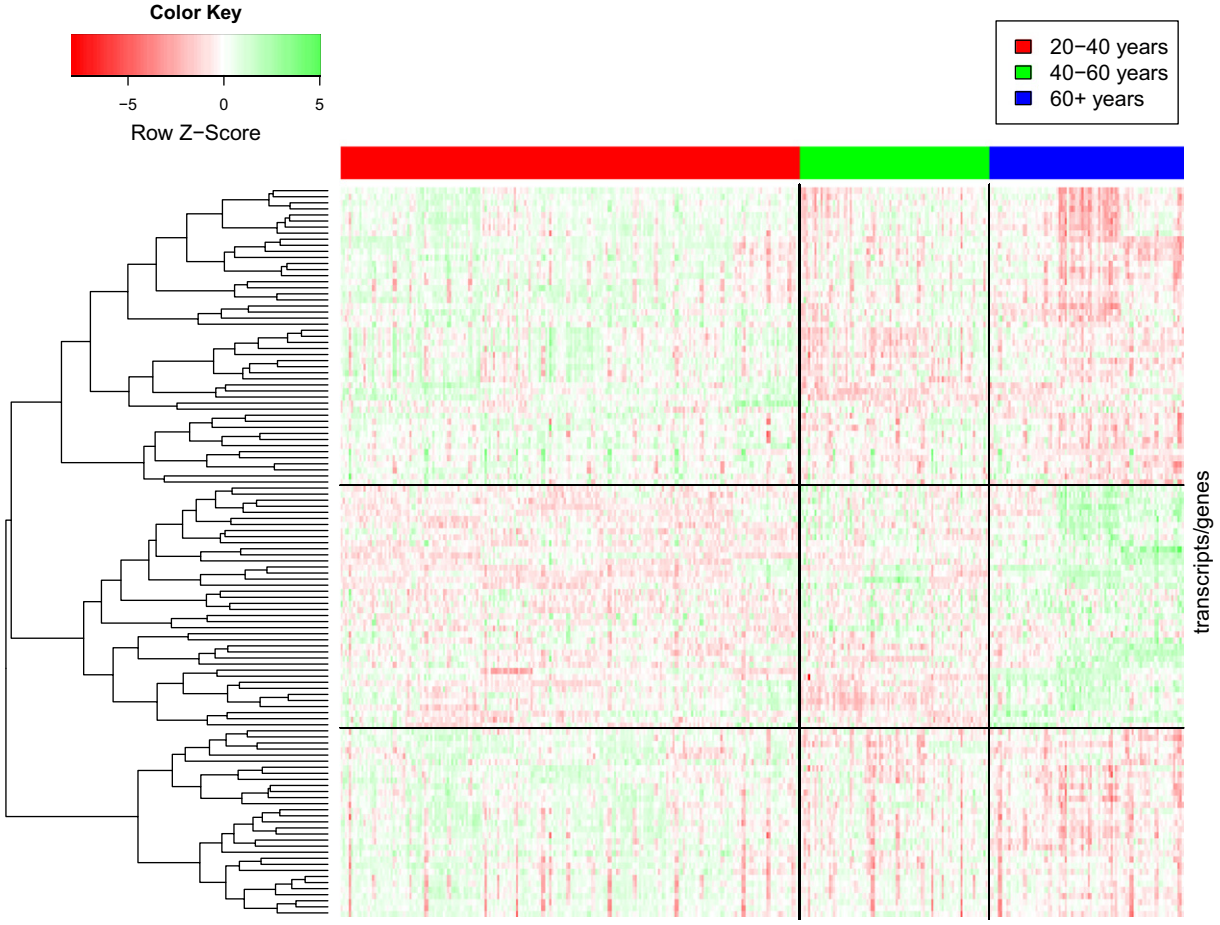

Fig. 2. Heat map for the expression levels of the 120 transcripts significantly altered in aging and PD in the Human Brain Transcriptome data set (Kang et al., 2011), comparing brain samples across three different age groups (20–40 years, 40–60 years and 60 + years; see also Fig. 1 showing the same transcripts in PD brain samples vs. controls). The plot uses a Pearson correlation hierarchical clustering (see dendrogram on the left and black horizontal lines, indicating the top-level cluster separations). A larger version of this map and an alternative clustering with the Euclidean distance metric for both genes and samples including all gene labels is provided in the Supplementary Material (details on the generation of the map are described in the Methods section).

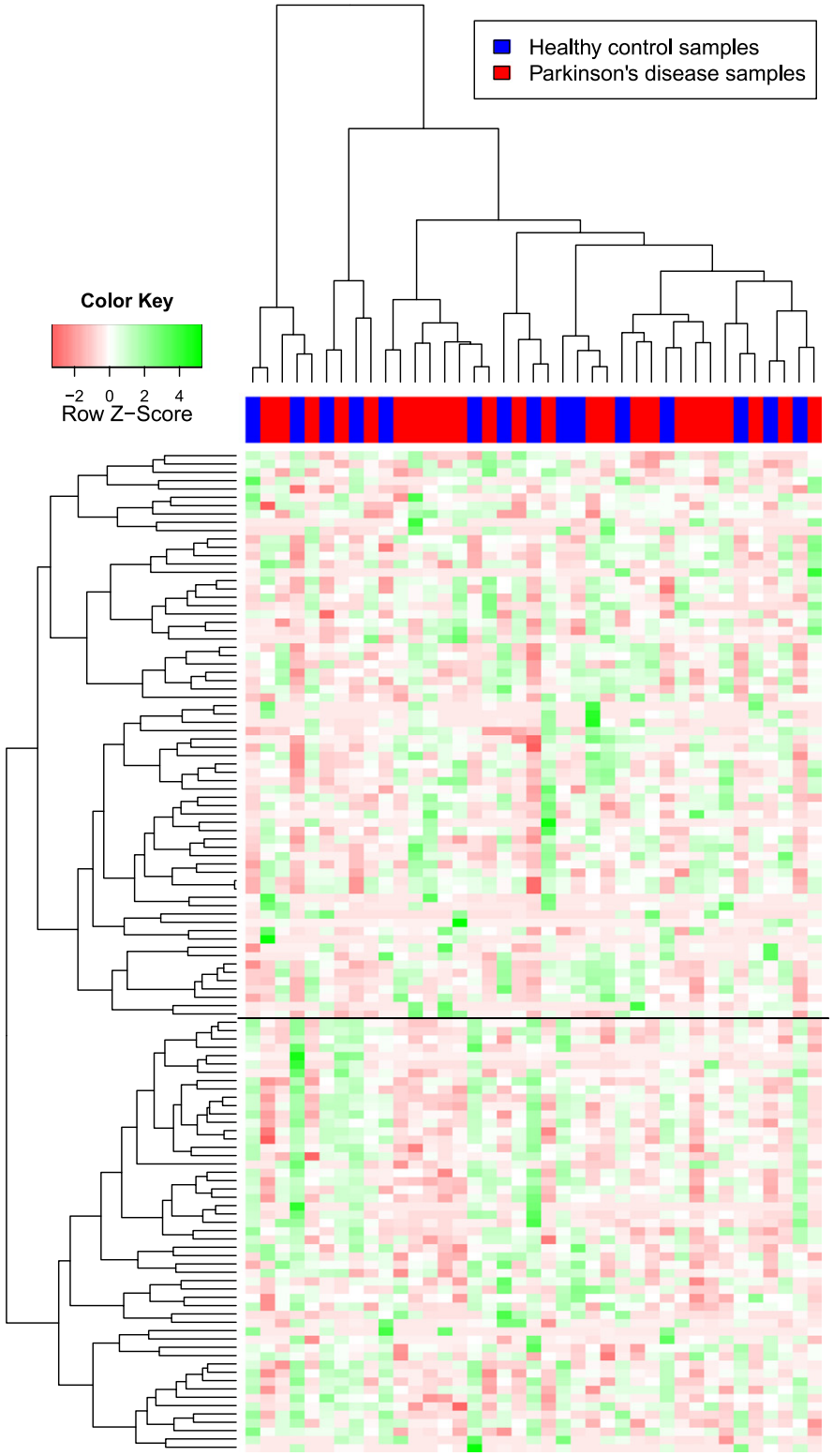

Fig. 3. Heat map for the expression levels of the 120 transcripts significantly deregulated in aging and PD in the data set by (Moran et al., 2006), comparing brain samples from the *substantia nigra* in Parkinson's patients (red) against controls (blue). A Pearson correlation distance metric was used to obtain the clustering for both rows and samples (the retained top-level cluster separation from Fig. 1 is indicated by the black horizontal line). The identified sample clusters do not coincide with the two known sample conditions (see color legend above). A larger version of this map and an alternative clustering with the Euclidean distance metric including all gene labels is provided in the Supplementary Material.

interest (e.g., reflecting differences in diet, lifestyle, etc.); (2) the implicit assumption of the clustering algorithm that a hierarchical structure exists among the samples is not fulfilled (while genes can often be - clustered into hierarchical categories of functionally similar genes, for samples from a case–control study with no family relationships between the participants a hierarchical structure cannot be expected a priori, and the differences between PD samples and controls may be gradual and form a continuous spectrum rather than segregating into discrete clusters); and (3) the complexity and heterogeneity of the high-dimensional data prevents the unsupervised clustering approach

Table 3
Significant network associations of deregulated genes in aging and PD with Gene Ontology biological processes.

GO term	Network association score (XD-score)	Significance of overlap *q*-value (Fisher's exact test)	Mapped pathway size	Overlap size
Dopamine metabolic process (GO:0042417)	1.61	0.38	14	4
Synaptic vesicle endocytosis (GO:0048488)	1.47	0.46	10	3
Positive regulation of synaptic transmission (GO:0050806)	1.47	0.46	10	3
Synaptic transmission, dopaminergic (GO:0001963)	1.46	0.60	13	3
Regulation of long-term neuronal synaptic plasticity (GO:0048169)	1.38	0.19	21	6
Positive regulation of endocytosis (GO:0045807)	1.36	0.33	13	4
Phosphatidylinositol metabolic process (GO:0046488)	1.27	0.16	19	6
Synaptic transmission (GO:0007268)	0.29	0.00067	300	38

from capturing specifically the clinically relevant patterns of interest and supervised analysis techniques which make use of the available sample class labels are required instead. In the remainder of the manuscript, we therefore focus on supervised analysis methods for gene ranking, pathway and network analysis, which do not depend on hierarchical structure assumptions. These methods also enable an assessment of the statistical significance, which is used to compare only the significant findings against the literature.

A first general comparison of the direction of gene alterations in both data sources shows that for the majority of genes, a down-regulation in the PD cases coincides with a negative correlation with aging, and correspondingly, a positive correlation with aging is observed more frequently for genes up-regulated in PD (with significant Pearson and Spearman correlations between the PD-related *Z*-scores and the correlations with aging of 0.43 and 0.36, respectively; permutation-based *p*-value < 0.001 in both cases).

Four of the shared significant genes, *NR4A2*, *CALB1*, *GRIA1* and *MAPT*, have been associated previously with PD and aging in independent studies. Among these, *NR4A2* (also known as *NURR1*) stands out as the most significantly differentially expressed gene (*Z*-score: −7.43) and for a high negative correlation with adult brain aging (−0.53, see box plot in Fig. 4 and statistics in Table 1). We therefore focus on the discussion of *NR4A2* and the literature findings relating to the other three genes are summarized in Table S9.

NR4A2 encodes a brain-specific transcription factor belonging to the nuclear receptor superfamily and controlling the expression of genes involved in the maintenance of the nervous system and dopamine metabolism (Sacchetti et al., 2006). Mutations and polymorphisms in this gene in familial cases of PD have been reported in multiple studies (Le et al., 2003; Xu et al., 2002; Zheng et al., 2003; Grimes et al., 2006; Liu et al., nd.; Sleiman et al., 2009), but these sequence variants only occur rarely and dedicated screening for them was unsuccessful in other cohorts (Zimprich et al., 2003; Nichols et al., 2004; Tan et al., 2004). PD-like molecular phenotypes were also observed in homozygous *NR4A2*-deficient mice, with a region-specific lack of dopaminergic neurons in the *substantia nigra* and ventrotegmental area (Le et al., 1999a). Moreover, in heterozygous knockout mice, reduced brain dopamine levels (Zetterström et al., 1997) and a significant decrease in locomotor activities as compared to age-matched wild-type mice have been reported (Jiang et al., 2005). A relation between *NR4A2* and natural human aging (independent of PD pathogenesis) had been proposed in a previous study showing that the number of *NR4A2*-immunoreactive nigral neurons is significantly reduced in middle-aged (23.13%) and aged (46.33%) individuals as compared to young subjects (Chu et al., 2002), in agreement with the results shown here. Interestingly, reduced *NR4A2* expression in heterozygous knockout mice has also been shown to increase the vulnerability of dopaminergic neurons to the neurotoxin MPTP, which induces Parkinsonism-like phenotypes, suggesting a neuroprotective role for the gene (Le et al., 1999b). More recently, down-regulation of *NR4A2* was shown to transcriptionally increase the expression of *alpha-synuclein* (Yang and Latchman, 2008), a gene for which mutations, duplications and triplications have been linked causally with PD and whose protein aggregation is considered as one of the main molecular hallmarks of PD (Ibanez et al., 2004; Chartier-Harlin et al., 2004; Fuchs et al., 2007).

Considering these observations in mice and humans in combination with the highly significant down-regulation of *NR4A2* in PD and during natural aging observed here, we hypothesize that an age-related decline of *NR4A2* brain expression levels in healthy individuals may increase the risk for developing PD independent of the presence of mutations or polymorphisms in this gene. A network analysis of the downstream effects of *NR4A2* under-expression in PD using manually curated regulatory

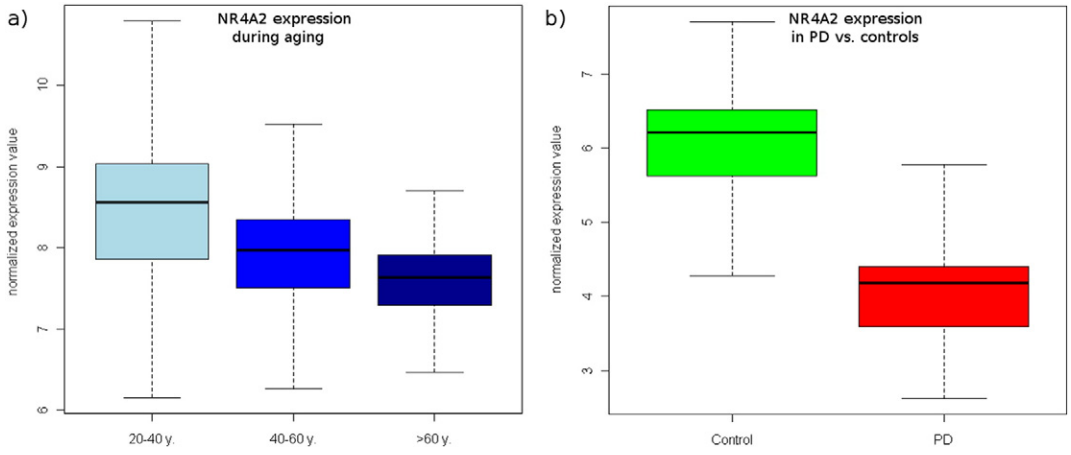

Fig. 4. (a) Box plot showing the median (bold horizontal line), interquartile range (box) and total range (whiskers) of normalized *NR4A2* expression levels across three different age groups and all brain regions in the HBT data set. (b) Box plot showing the median (bold horizontal line), interquartile range (box) and total range (whiskers) of normalized *NR4A2* expression values across closely age-matched PD patients and healthy controls in the data set by Moran et al. (Moran et al., 2006) (median age PD cases: 81, median age control cases: 77). Both expression alterations shown in a and b are statistically significant (FDR < 0.05).

interactions from the ResNet database (Nikitin et al., 2003) confirmed a significant down-regulation of direct target genes involved in dopamine metabolism, matching with previous observations for *NR4A2* mutations and knockout mice (see downstream network in Fig. S9). Moreover, when comparing genes differentially expressed in midbrain dopaminergic neurons of *NR4A2* knockout mice as opposed to wildtype mice (Kadkhodaei et al., 2013) against the significantly altered genes in PD from the meta-analysis of human brain samples, 45 shared significant genes are found (see Table S4), including the known direct *NR4A2* targets *TH* and *SLC18A2*, which are markedly under-expressed in both data sets. In total, 32 genes display shared significant down-regulation in the knockout model and the PD meta-analysis, one gene, *DDX3Y*, is jointly up-regulated and 13 genes show opposite alterations, potentially resulting from more complex multifactorial and indirect regulatory mechanisms (see Table S4). Interestingly, the jointly down-regulated genes contain the mitochondrial complex I genes *NDUFB2* and *NDUFB8*, suggesting a possible mechanistic link between NR4A2 dysregulation and mitochondrial dysfunction in Parkinson's disease for further study (*NDUFB2* expression in the brain is also altered during adult aging in the HBT data set, displaying an up-regulation with increasing age).

Next, in order to narrow down potential upstream regulatory causes for the observed age-dependent down-regulation of *NR4A2*, a network analysis was applied to *NR4A2* upstream regulators. This analysis identified a significant down-regulation of CREB-dependent gene transcription with increasing age as putative upstream cause for the age-associated decline in *NR4A2* expression levels (see the network visualization shown in Fig. 5). Since CREB activation mediates mitochondrial gene expression and survival in response to mitochondrial dysfunction (Arnould et al., 2002; Lee et al., 2005), the down-regulation of CREB regulator genes with increasing age also matches with observed age-associated alterations in mitochondrial processes found in the analysis of gene groups with shared molecular functions (see corresponding section below).

In summary, the 120 shared deregulated genes in aging and PD display significant correlation in terms of the direction of their expression changes (with a majority of up- and down-regulation patterns occurring jointly in higher age groups and in PD samples vs. controls) and include four genes that have been implicated in PD and aging in multiple independent studies (*NR4A2*, *MAPT*, *GRIA1* and *CALB1*). For the observed under-expression of *NR4A2*, the combination of transcriptome network analyses, mouse knockout data analysis and previous findings from the literature provide details on possible upstream causes and downstream effects, suggesting in particular that *NR4A2* has a regulatory influence on the expression levels of alpha-synuclein and mitochondrial complex I genes.

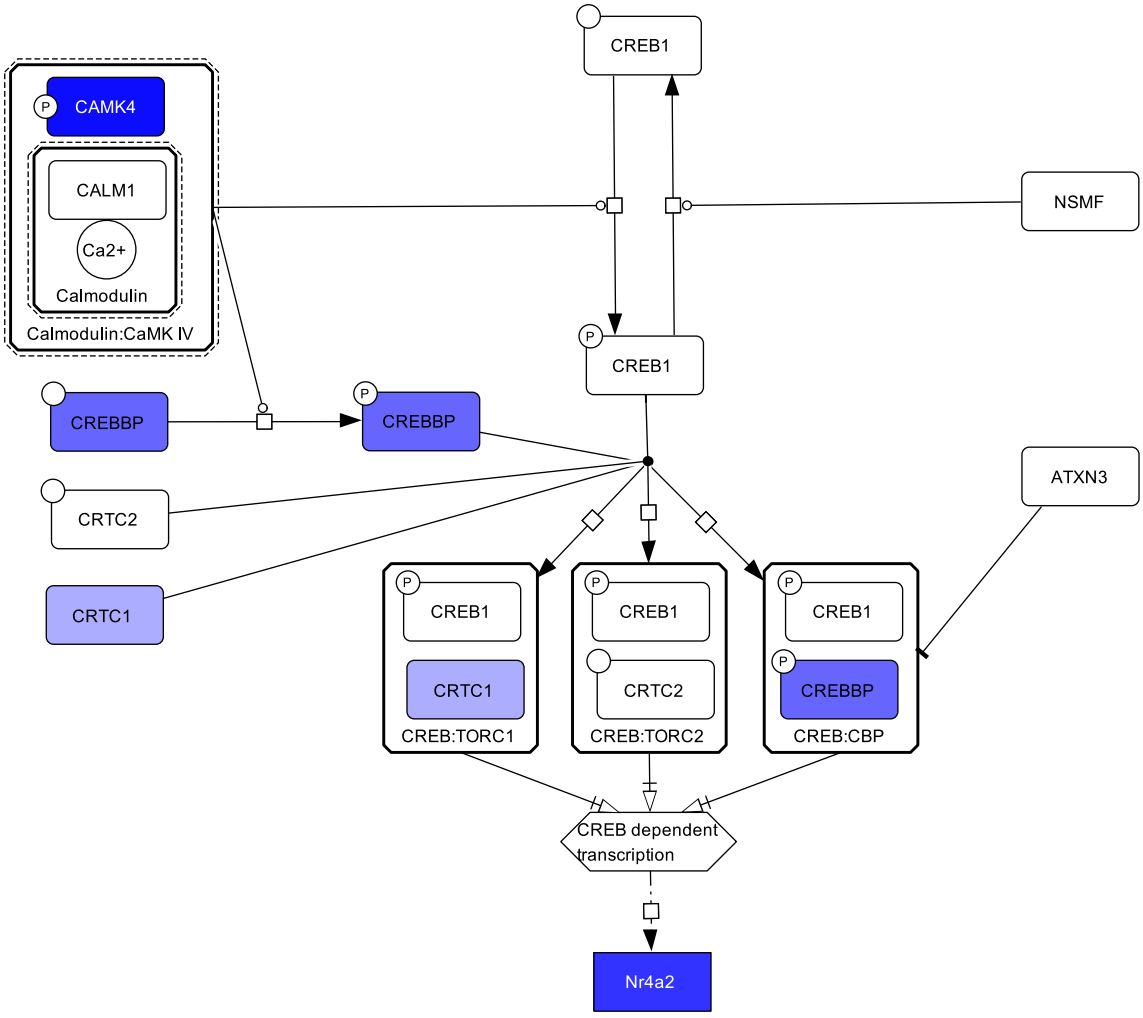

Fig. 5. Upstream regulatory network for NR4A2 revealing down-regulated genes in aging associated with regulation of CREB-dependent gene transcription (blue nodes, the color darkness is proportional to the absolute ***Z*-score** of differential expression). A more comprehensive interactive cellular pathway map for Parkinson's disease map can be explored online using different color-overlays for deregulated genes in aging and in PD (http://minerva.uni.lu/MapViewer/map?id=pdmap-ageing).

Analysis of peripheral expression and disease specificity of gene alterations for biomarker applications

Apart from using the identified jointly deregulated genes in PD and aging to analyze the age-dependence of PD risk at the molecular level, these genes may also serve as candidates for developing new risk biomarkers for PD or general neurodegeneration. The rationale is that the jointly deregulated genes are more likely to reflect age-dependent contributions to the disease risk than genes altered only in PD, and their expression levels could therefore mark an increased risk already before the onset of motor symptoms. Future validation studies will however first need to identify peripheral surrogate markers to assess gene deregulations in the brain indirectly, e.g., by screening for correlated gene or protein expression levels in cerebrospinal fluid, blood or saliva. Previously, similar approaches have been applied successfully to other neurological disorders, e.g., a blood biomarker for schizophrenia has been derived from a comparative gene expression analysis of brain and blood samples (Glatt et al., 2005). We have therefore used tissue-specific gene expression data from the public GNF Gene Expression Atlas (Su et al., 2004) to provide a tabular overview of the extent to which the 120 jointly altered genes in PD/aging are expressed in peripheral blood and across different non-brain tissues as compared to brain tissues (see Table S5). For the majority of genes, the median expression in peripheral tissues is similar or higher in relation to the median expression in the brain. Table S5 also highlights the genes/proteins for which altered activities in PD or aging have been reported previously in peripheral body fluids and tissues, including NR4A2 and the metallothioneins MT1H and MT1G (see also the gene group analysis in the following section).

Moreover, to examine the specificity of the jointly altered genes for PD as compared to a different neurodegenerative disorder, the same normalization procedure and test statistic was applied to determine the genes' differential expression in Alzheimer's disease (AD) brain transcriptomics data, covering 387 annotated AD samples and 300 controls (Zhang et al., 2013). Although the comparability of the PD and AD transcriptomics data is necessarily limited due to differences in the brain regions considered as the main sites affected (*substantia nigra* in PD vs. *hippocampus* in AD) and the lack of a matching between progression stages in the two diseases, 95 of the 120 jointly altered genes in PD/aging were also significantly differentially expressed in AD, and 85 of these altered in the same direction (up/down) as in PD (corresponding to an overall matching of 70.8%, see Table S6). While the shared significant genes in PD, AD and aging provide possible candidates for general biomarkers of neurodegeneration, the genes with diverse alteration direction in PD as compared to AD and aging (e.g., *NPAS2* and *EXT1*) could be of interest for the development of PD-specific markers. However, in order to obtain robust biomarker models, further validation of these candidate genes/proteins and their activity profiles in peripheral tissues will be required on large-scale independent cohorts, including the investigation and testing of multifactorial marker models.

Joint PD/aging-related expression changes in groups of genes with shared molecular functions

In order to facilitate the biological interpretation of newly identified genes with joint alterations in aging and PD, we have grouped them according to their shared molecular function annotations (see Table 2). A further goal was to determine whether the intersection between late-stage PD-deregulated genes and aging-associated genes would be enriched in cellular processes implicated in the early/prodromal-stage and progression of the disease rather than mainly representing late-stage downstream effects. Since aging is considered as a major risk-promoting factor for PD, joint aging/PD-associated molecular alterations would be expected to occur already in the early and presymptomatic stages of PD. Thus, interlinking the joint transcriptome changes with pathway alterations implicated in the initial phases of PD could help to reduce the need for scarcely available prodromal-stage data in the search for early stage diagnostic biomarkers.

Overall, the identified processes affected by joint PD/aging expression changes largely match with cellular processes previously proposed to be linked with the early phases of PD pathogenesis, including mitochondrial and lysosomal processes, apoptosis and processes associated with neuron differentiation and inflammation (McGeer and McGeer, 2004; Pan et al., 2008; Shadrina et al., 2010). The observed mitochondrial alterations are in line with previous discoveries of disease-causing mutations in familial cases of PD, affecting the mitochondrial genes *DJ1*, *PINK1*, *PARK2* and *HTRA2* and supporting the hypothesis of an involvement of mitochondrial dysfunction in the early stages of idiopathic PD (Abou-Sleiman et al., 2006; Büeler, 2009). Moreover, pesticides and neurotoxins acting at mitochondrial complex I have been described to induce Parkinsonism-like symptoms (Sherer et al., 2007). In our study, we observe significant alterations in the gene for complex I subunit *NDUFB2* in both aging and PD. Among the 45 complex I subunits, *NDUFB2* functions as an NADH dehydrogenase and oxidoreductase and is involved in the transfer of electrons from NADH to the respiratory chain.

Similar to mitochondrial dysfunction, defects in the lysosome/autophagy pathway (ALP) have also been considered as possible causes of PD and other neurodegenerative diseases (see (Pan et al., 2008) for example). Interestingly, among the lysosomal genes found jointly altered in PD and aging here, the gene *SMPD1* can harbor different rare variants, which have recently been proposed as risk factors for PD in different studies (Gan-Or et al., 2013; Foo et al., 2013).

A causal role of inflammation in PD is more controversially discussed. While inflammatory responses are observed in many diseases and often regarded as a purely secondary effect, more recently, neuro-inflammatory processes have been investigated as possible causative or contributing factors in PD. For example, microglial overactivation is thought to result in the loss of dopaminergic neurons in PD patients (Qian et al., 2010) and large numbers of human leukocyte antigens (HLA-DR) and CD11b-positive microglia have been detected in the *substantia nigra* brain region in patients (McGeer et al., 1988). The down-regulated transcription factor *NR4A2*, discussed in detail above, has been shown to act as a repressor of genes encoding pro-inflammatory neurotoxic factors in microglia and astrocytes, protecting dopaminergic neurons from inflammation-induced death (Saijo et al., 2009). A further inflammation-associated regulator detected as significantly under-expressed in PD and with increasing age is attractin (*ATRN*), involved in the initial immune cell clustering during the inflammatory response. Attractin overexpression has been shown to protect mitochondrial function in animal studies with Parkinsonism-inducing toxins, and an aging-dependent decrease in *ATRN* expression has also been observed in mice (Paz et al., 2007).

While most significantly deregulated processes in aging and PD presented in Table 2 cover both up-and down-regulated genes, a consistent joint up-regulation in PD and positive correlation with aging was found for a group of three genes involved in metal ion homeostasis. They include two metallothioneins (MTs), *MT1G* and *MT1H*, encoding metal-binding proteins known to be involved in the cellular response to metal ion toxicity, oxidative stress and inflammation (Andrews, 2000). MTs have been shown to prevent oxidative stress and attenuate apoptosis, and experiments on animal models suggest that MTs also promote neuronal survival and regeneration *in vivo* (Sharma and Ebadi, 2011; Ambjørn et al., 2008). Activation of MTs has also been proposed as a strategy to inhibit neurodegenerative alpha-synucleinopathies like PD, and MT activity profiles have been suggested as early stage markers for neurodegeneration (Sharma and Ebadi, 2011). Apart from the identified jointly deregulated MTs in PD and aging, we found further MTs significantly up-regulated in the PD transcriptomics samples (*MT1M*, *MT1F*, *MT1P2*, *MT1X*, *MT2A*, *MT3*, *MT4*, as well as the transcription factor *MTF1* regulating MT expression), while no MT transcript

was significantly down-regulated, confirming the potential of general MT over-expression as a robust multigene biomarker for PD or general neurodegeneration. Support for an involvement of MTs in neurodegeneration was also obtained by the analysis of PD-associated GWAS data (see corresponding section below).

Apart from investigating pre-defined gene groups with shared functional annotations, we also applied a differential co-expression analysis (Watson, 2006) to identify new groups of co-expressed genes among the jointly altered genes in PD and aging, whose co-expression pattern changes in PD as compared to controls. However, no significant co-expression pattern identified on individual transcriptomics data sets was found replicated across the data from other PD case–control studies (only the metallothioneins MT1G and MT1H were consistently co-expressed across different data sets, matching with the observed up-regulation of the transcription factor *MTF1* which co-regulates their expression, see above). To identify more complex gene regulatory mechanisms, the enrichment of transcription factor binding sites (TFBS) among the shared significantly altered genes in PD/aging and all significant genes in PD was assessed using the F-Match algorithm (BioBase Explain 3.0 software, (Kel et al., 2006), see Tables S7 and S8). In both analyses, a binding site targeted by the transcription factor *FOXO4* was top-ranked among the significant results. *FOXO4* is also significantly up-regulated in the PD meta-analysis (*Z*-score 5.4) and known to up-regulate superoxide dismutase-2 in response to oxidative stress (Araujo et al., 2011). A further significant TFBS in both analyses is targeted by *PATZ1*, a gene significantly up-regulated in PD (*Z*-score 3.4) previously found to inhibit endothelial cell senescence and to be involved in the regulation of reactive oxygen species levels (Cho et al., 2011). Additional experimental studies will be required to confirm the involvement of these regulatory genes in PD.

In summary, the main functional annotations represented among the 120 jointly deregulated genes in PD and aging point to shared alterations in cellular processes that have previously been implicated in the disease and in particular the early stages of PD pathogenesis. Aging-related activity changes in these processes and upstream transcription factors may therefore contribute to the age-dependence of PD risk, and some of the most robust among these changes (e.g., the pronounced up-regulation of several metallothioneins) for genes which are also expressed in peripheral tissues could serve as a basis for developing multifactorial biomarker models.

Jointly deregulated pathways and network modules in Parkinson's disease and brain aging

To obtain a pathway-level statistical assessment of shared transcriptome changes in aging and PD, we use a network-based pathway analysis approach, which also enables a visual analysis of the underlying network for top-ranked associations between known cellular pathways and differentially expressed genes. As a complement to a classical pathway enrichment analysis (scoring the significance of the overlap between members of cellular pathways and deregulated genes from a microarray study), network-based association statistics can identify new significant interrelations between gene/protein sets with small or no overlap. For this purpose, a statistical test assesses whether the mapped gene/protein sets are more densely and closely interconnected in a protein–protein interaction network or gene regulatory network than expected by chance according to a random background model (see Methods). Thus, for the pathway-level analysis of PD/aging transcriptome alterations, we mapped all significantly differentially expressed genes (FDR < 0.05) from the aging data set and the matched-size top-ranked genes from the PD cross-study analysis (again with FDR < 0.05) onto a genome-scale protein–protein interaction network, containing only experimentally verified, direct physical interactions assembled from public databases (Bossi and Lehner, nd.). Next, we scored the associations of the mapped genes with known cellular pathways from public databases (Gene Ontology (Ashburner et al., 2000), KEGG (Kanehisa and Goto, 2000), WikiPathways (Pico et al., 2008) and Reactome (Joshi-Tope et al., 2005) using both a conventional enrichment analysis (Fisher's exact test) and our previously developed graph-based statistic, implemented in the public web-application *EnrichNet* (see Glaab et al., 2012 and Methods for details).

Table 3 shows the biological processes from the Gene Ontology (GO) database which were identified to have statistically significant over-representation or network association scores with the deregulated genes in PD and aging (network association was measured in terms of the XD-score as defined in Glaab et al., 2012, which assigns higher scores to more significant associations, and pathway over-representation was measured using Fisher's exact test and *q*-value false-discovery rate scores; see Supplementary Material for the results on other pathway databases). The first seven table entries contain the processes significant in terms of the network association score, and the last entry (*synaptic transmission process*, GO:0007268) represents the only process significant in terms of the over-representation analysis (*q*-value < 0.05).

Overall, 5 of the 8 GO biological processes scored to have significant associations with the aging- and PD-deregulated genes are synaptic processes (*synaptic vesicle endocytosis*, *positive regulation of synaptic transmission*, *dopaminergic synaptic transmission*, *regulation of long-term neuronal synaptic plasticity* and general *synaptic transmission*), suggesting that they belong to those most profoundly affected by PD- and aging-related gene alterations. Among the other significant pathways, the top-ranked GO-term *dopamine metabolic process* matches with the known decline in dopamine synthesis observed during natural aging and PD pathogenesis (see Introduction). Moreover, two of the identified significant GO-terms point to associations of the deregulated genes with endocytic processes (*positive regulation of endocytosis* and *synaptic vesicle endocytosis*). Since *LRRK2*, the most commonly mutated gene in familial cases of PD, is known to regulate synaptic vesicle endocytosis (Shin et al., 2008), and aggregation of α-synuclein as one of the major neuropathological hallmarks of PD was found to be associated with defects in endosomal trafficking in a *Saccharomyces cerevisiae* model for PD (Soper et al., 2011), these observations warrant further study of endocytic pathway alterations as a functional link between aging and PD (see also (Blanpied et al., 2003; Nixon, 2005) for a discussion of age-related changes in endocytosis and endosome dysfunction in neurodegenerative diseases).

An entirely novel association between brain aging processes and molecular changes in PD is suggested by the significant network association of the deregulated genes with the GO-term *phosphatidylinositol metabolic process*. This matches with an aging-related decline in the activity of this pathway observed in rats and the known role of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in controlling the survival of neurons (Chae and Kim, 2009).

To investigate the identified pathway associations in more detail, we created visualizations of the sub-networks interlinking the aging/PD-deregulated genes with the cellular processes. Figs. S10–S17 show corresponding sub-networks for the top-ranked GO terms. These visualizations reveal, for example, that two interaction partners of the familial PD-associated alpha-synuclein protein (*SNCA*) are significantly altered during adult aging, *MAPT* (see previous discussion above) and the SNCA-degrading serine protease *KLK6*.

Apart from using generic pathway databases, we have mapped the significant genes in aging and PD onto a cellular process map specific for Parkinson's disease and obtained from manual curation of the literature in collaboration with the Systems Biology Institute in Tokio, Japan (Fujita et al., 2014). An interactive version of this map has been made publicly available for online exploration, including different color-overlays to highlight the deregulated genes in aging and PD identified in our study (http://minerva.uni.lu/MapViewer/map?id=pdmap-ageing).

Table 4
Genes containing PD-associated SNPs with significant differential expression in both Parkinson's disease and adult brain aging (GWAS findings with significance $p < 10E-08$ are highlighted in bold).

Genesymbol (HGNC)	Gene description	GWAS p–value	Deregulation in PD samples (Z–score)	Correlation with aging
MAPT	microtubule–associatedproteintau	**7E–12**	−2.88	−0.51
PLEKHM1	pleckstrinhomologydomaincontaining, familyMmember1	**6E–08**	4.05	−0.41
MT1G	metallothionein1G	4.2E–05	4.31	0.57
CAMTA1	calmodulinbindingtranscriptionactivator1	0.0001	−4.2	−0.44
FAM49A	familywithsequencesimilarity49,memberA	0.0005	−4.99	−0.45
ARHGEF10	Rhoguaninenucleotideexchangefactor(GEF)10	0.0009	4.57	−0.25

Enrichment analysis of PD-associated sequence variants among aging-/PD-deregulated genes

Apart from the contribution of aging to the risk of developing PD and different environmental risk factors, the disease is also thought to have a major genetic component. Familial cases of PD with known causative mutations currently account for only 5% to 10% of patients (Lesage and Brice, 2009); however, the patients' family history and genome-wide association studies (GWAS) suggest that a significantly larger number of genetic variations alters the disease risk (e.g., a recent large-scale GWAS estimated the heritability of PD to be at least 0.27 Do et al., 2011). As an additional exploratory analysis, we therefore investigated a possible enrichment of genes with PD-associated sequence variants from published GWAS reported in the Ensembl Variation database (Chen et al., 2010) among the genes with significantly altered expression in PD and aging (see Methods section for details on data collection). For this purpose, we assigned the SNPs to two groups depending on their significance: (1) SNPs with genome-wide significance (defined as $p < 10E-08$) and (2) suggestive SNPs (defined as $p < 0.001$).

From the set of 325 unique genes with suggestive PD-linked SNPs obtained from the Ensembl database, we found 6 genes (2 among them with genome-wide significance) overlapping with the significantly altered genes in PD and aging ($p = 0.03$ for the overlap with the suggestive set and $p = 0.01$ for the overlap with the genome-wide significant set; Fisher's exact test). These include the already discussed genes tau (*MAPT*) and metallothionein 1G (*MT1G*), but also four new candidate disease genes for further investigations: *FAM49A*, *ARHGEF10*, *CAMTA1* and *PLEKHM1* (see Table 4, a detailed discussion for each of these genes in the context of PD is provided in the final section of the Supplementary Material). Since the available transcriptome data were not complemented by corresponding sequence variant data for the same samples to assess a direct relation between genome and transcriptome alterations, verification of the candidate genes derived from this exploratory analysis will require validation on independent complementary genetic and transcriptome data.

Conclusions

The joint analyses of brain gene expression in natural aging and PD reveal significant shared individual gene and cellular pathway alterations. Apart from the observed changes in processes implicated in aging and PD pathogenesis, including mitochondrial dysfunction, disruption of lysosomal/autophagic function and apoptosis, neuroinflammation and metal ion homeostasis, we find previously unreported significant joint process alterations, e.g., affecting synaptic vesicle endocytosis and phosphatidylinositol metabolism. Complementing the identification of significant genes and pathways, subsequent network, literature mining and knockdown data analyses for *NR4A2* as the most significantly under-expressed gene in PD among the jointly altered genes also suggest a mechanistic explanation of how the down-regulation of *NR4A2* with increasing age may increase PD risk via reduced expression of dopamine transporters and mitochondrial genes and an up-regulation of alpha-synuclein. These results match with the dopamine-depleting effects observed in *NR4A2* knockout mice and the human PD-associated mutations reported in this gene.

Since aging is regarded as one of the main risk factors for PD, the most robust gene and process deregulations among the shared significant alterations also provide specific candidates for building early stage risk biomarker models for PD or general neurodegeneration. This task will require further research and experimental validation and may be achieved by screening for correlated surrogate markers in cerebrospinal fluid, blood or saliva.

Finally, the combined analysis approach for aging and PD proposed here may serve as a template for applying similar integrative analyses to other neurodegenerative diseases in order to study aging-related commonalities between these disorders.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.11.002.

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