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Common mechanisms in neurodegeneration and neuroinflammation: a BrainNet Europe gene expression microarray study

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Abstract Neurodegenerative diseases of the central nervous system are characterized by pathogenetic cellular and molecular changes in specific areas of the brain that lead to the dysfunction and/or loss of explicit neuronal populations. Despite exhibiting different clinical profiles and selective neuronal loss, common features such as abnormal protein deposition, dysfunctional cellular transport, mitochondrial deficits, glutamate excitotoxicity, iron accumulation and inflammation are observed in many neurodegenerative disorders, suggesting converging pathways of neurodegeneration. We have generated comparative genome-wide gene expression data, using the Illumina HumanRef 8 Beadchip, for Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, multiple sclerosis, Parkinson's disease, and schizophrenia using an extensive cohort ($n = 113$) of well-characterized post-mortem brain tissues. The analysis of whole-genome expression patterns across these major disorders offers an outstanding opportunity not only to look into exclusive disease-specific changes, but more importantly to look for potential common molecular pathogenic mechanisms. Surprisingly, no dysregulated gene that passed our selection criteria was found in common across all six diseases. However, 61 dysregulated genes were shared when comparing five and four diseases. The few genes highlighted by our direct gene comparison analysis hint toward common neuronal homeostatic, survival and synaptic plasticity pathways. In addition, we report changes to several inflammation-related genes in all diseases. This work is supportive of a general role of the innate immune system in the pathogenesis and/or response to neurodegeneration.

Keywords Microarray · Neurodegeneration · Neuroinflammation · Microglia · Astrocytes · Glia reactivity

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Introduction

Despite the development of drugs that improve both symptoms and quality of life for people diagnosed with neurodegenerative disorders (NDs) such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS), none of them are effective at preventing the progressive neuronal loss and the consequent accumulation of neurological symptoms and disability. Furthermore, the development of new innovative central nervous system (CNS) drugs is slow (Pangalos et al. 2007), thus giving impetus for novel research aimed at understanding the underlying pathogenesis of neuronal cell dysfunction and death in these disorders.

Central nervous system's neurodegenerative disorders are in general characterized by cellular and molecular pathological changes in disease-specific areas of the brain that lead to the dysfunction and/or loss of specific neuronal populations. Despite showing different clinical profiles and selective neuronal loss, common features such as abnormal protein deposition, abnormal cellular transport, mitochondrial deficits, and iron accumulation and glutamate excitotoxicity are observed to a varying extent in most of the disorders, suggesting converging pathways of neurodegeneration. Inflammation is another common feature believed to contribute to a differing extent to the pathogenesis of a broad spectrum of neurodegenerative disorders (Amor and Woodroofe 2014) and the exact involvement of glia and the immune system in neuronal cell death remains to be fully understood. Glial support and homeostasis is not always maintained and reactive astroglia and/or microglia have been implicated in the pathogenesis of all the major neurodegenerative disorders (Aronica et al. 2001; Cagnin et al. 2001; Magliozzi et al. 2010; Pavese et al. 2006; Yiangou et al. 2006).

The use of transcriptomics and proteomics has identified novel key molecules that have provided further mechanistic insight into neurodegeneration by identifying both pro-apoptotic and neuroprotective signaling pathways (Altar et al. 2009; David et al. 2005). Gene expression profiling has permitted, for example, the identification of dynactin-1 as a causative gene for ALS (Jiang et al. 2005) and osteopontin (Lock et al. 2002) as important in the pathogenesis of MS. Microarray studies have also extended the complex gene expression patterns and confirmed the involvement of multiple cellular pathways in AD (Reddy and McWeeney 2006) and in PD (Grunblatt et al. 2004). Numerous microarray studies have generated gene expression profiles for major CNS disorders. However, lack of comparability and reproducibility between the different microarray platforms has raised doubts about the validity of this approach, despite constant efforts at standardization (Shi et al. 2006). Comparisons across different diseases proved almost impossible due to differences in sensitivity between microarray platforms, to variation in oligonucleotide probe length, in protocols used for sample preparation and/or in statistical tests used for data analysis (Roberts 2008). Only a few attempts have been made so far to apply a genomic approach to a cross-disease comparison of transcriptional profiles. A recent study using the Affymetrix GeneChip compared different brain regions from Alzheimer's and Parkinson's disease cases and found twelve genes dysregulated in a similar manner in both diseases (Grunblatt et al. 2007). Complex pathogenetic pathways have also been compared for MS-, AD-, and HIV-associated dementia based on findings from lipidomics, proteomics, transcriptomics, and genomics studies (Noorbakhsh et al. 2009). Although intrinsic obstacles still exist, microarray technology is constantly evolving to compensate for its limitations.

As a result of the formation of a European brain banking network, BrainNet Europe (BNE), that has standardized protocols for the collection, storage, and characterization of human post-mortem materials (Bell et al. 2008; Durrenberger et al. 2010; Kretzschmar 2009), we have been able to carry out an extensive and unique analysis of genome-wide changes in gene expression in AD, ALS, HD, MS, PD, and schizophrenia (SZ) using a unified approach. To minimize experimental biases, this study was conducted using the same optimized protocol for sample preparation, the same array platform (the Illumina whole-genome HumanRef8 v2 BeadChip) and the same software for the data

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normalization and the differential gene expression analysis (Rosetta Resolver® System). Our main aim was to identify common genes responsible for the neuronal and glial pathology observed in neurodegenerative disorders using microarray technology.

Materials and methods

Tissue samples

A total of 113 cases were selected retrospectively on the basis of a confirmed clinical and neuropathological diagnosis, including the exclusion of confounding pathologies, and snap-frozen brain blocks were provided by various tissue banks within the BNE network (Barcelona, Budapest, Goettingen/Mannheim, London Imperial College, Munich and Würzburg). The respective control tissues were matched for age, post-mortem (PM) delay and brain region, came from the same brain banks and in addition from the Human Brain Tissue Bank in Budapest (M.P.). Human brain reference RNA was purchased (Applied Biosystems, Foster City, CA, USA). The basic clinical and neuropathological characteristics can be found in the electronic supplementary resources (Online Resource 1) and a summary is presented in Table 1. Ethical committee permission for the collection of the tissues was in place in each individual brain bank, according to local national regulations.

To study the mechanisms involved in neuronal cell death in the different disease states, we chose tissue blocks representing CNS regions and disease stages in which we would have expected to observe ongoing pathology as a result of the primary disease mechanisms. For AD, we chose the entorhinal cortex from 12 cases at Braak stages IV (5) and V (7). For ALS, we chose to study the cervical cord from nine cases. For HD, we chose tissue blocks containing the ventral head of the caudate nucleus from 10 cases. For MS, we dissected subpial grey matter lesions from tissue blocks from the frontal gyri from 10 cases with a relatively young age at death (49.4 ± 4.6). For PD, we chose the substantia nigra from 12 cases with disease duration mean of 13 years (range 5–24). For schizophrenia (SZ), due to a marked loss of grey matter in Brodmann area 22 (BA22) (Hazlett et al. 2008), we chose tissue blocks from the left BA22 of the superior temporal cortex from 10 well-diagnosed chronic schizophrenia patients. Schizophrenia was included in this study as an example of a chronic CNS disorder without substantial neuronal loss and classical signs of a neurodegenerative process. All schizophrenia patients had been long-term in-patients at the Mental State Hospital Wiesloch, Germany, and the

Table 1 Summary of basic demographic data of cases

Samples	N	Brain area/tissue bank/ (BNE participant)	M	F	Age at death	PMD	RIN
AD:disease	12	Entorhinal cortex/Institute of Neuropathology in Barcelona (I.F.)	7	5	81.33 (±5.35)	6.00 (±3.5)	7.31 (±0.66)
AD:control	6		3	3	60.33 (±14.19)	8.70 (±13)	7.25 (±0.69)
ALS:disease	9	Cervical spinal cord/Raymond Escourolle Neuropathology Laboratory, Paris (D.S.)	6	3	68.11 (±8.49)	28.11 (±5.82)	6.38 (±0.59)
ALS:control	7		7	0	63.86 (±18.48)	13.87 (±11.36)	6.61 (±0.93)
HD:disease	10	Ventral head of the caudate nucleus/Institute of Neuropathology in Munich (T.A.) and from University of Würzburg (E.G.)	7	3	59.11 (±11.36)	18.20 (±13.14)	6.81 (±1.09)
HD:control	10		8	2	53.70 (±15.71)	21.29 (±6.26)	8.19 (±1.08)
MS:disease	10	Subpial GM lesions in the frontal gyri/UK MS Tissue Bank at Imperial College London (R.R.)	5	5	49.40 (±4.58)	16.80 (±7.63)	7.54 (±0.57)
MS:control	10		6	4	53.10 (±4.25)	11.49 (±7.78)	7.20 (±0.45
PD:disease	12	Substantia nigra/UK PD Tissue Bank, Imperial College London (D.D.) and the University of Würzburg (E.G.)	6	6	81.50 (±3.73)	38.89 (±6.64)	6.84 (±0.53)
PD:control	7		5	2	65.86 (±17.38)	21.64 (±14.76)	6.31 (±0.69)
SZ:disease	10	Left temporal lobe (BA22)/ Department of Psychiatry, University of Göttingen (P.J.G-H.)	5	5	66.30 (±11.97)	20.80 (±9.94)	6.52 (±0.88)
SZ:control	10		5	5	61.20 (±14.55)	17.33 (±5.84)	6.43 (±1.12)

(*M* male; *F* female; Mean ± SD)

diagnosis of schizophrenia was made prior to death by an experienced psychiatrist according to the Diagnostic and Statistical Manual of Mental Disorders IV criteria. Tissues from SZ patients and controls underwent neuropathological characterization to rule out associated neurovascular or, in the case of controls, neurodegenerative disorders (<Braak stage II) (Braak et al. 2006). None had a history of alcohol, drug abuse, or severe systemic illness.

Total RNA extraction

Total RNA was extracted from dissected snap-frozen tissue (<100 mg) by the individual laboratories according to an identical BNE optimized common protocol (Durrenberger et al. 2010) using the RNeasy® tissue lipid mini kit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions, and was stored at −80 °C until further use. RNA concentration and purity was assessed by spectrophotometry (NanoDrop ND1000; NanoDrop Technologies, Delaware, USA) and RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies UK Ltd, West Lothian, UK). All staff carrying out the RNA extractions had been previously trained in the protocol at Imperial College London.

Microarray hybridization and analysis

Gene expression analysis was performed on the RNA samples using the Illumina whole-genome HumanRef8 v2 BeadChip (Illumina, London, UK). Samples were profiled on more than 24,000 probes annotating more than 20,500 genes. This platform was chosen for its advantageous performance with 100 test genes. All the labeling and hybridization of the samples ($n = 120$; including technical replicates and human reference) was carried out in a single experiment by the Imperial College group to reduce the technical variability. RNA samples were prepared for array analysis using the Illumina TotalPrep™ 96 RNA Amplification Kit (Ambion/Applied Biosystems, Warrington, UK). First and second strand complementary DNA was synthesised from 0.5 μg of total RNA and purified. Complementary RNA was then synthesised, purified, and labeled with biotin. The biotin-labeled complementary RNA was applied to the arrays using the whole-genome gene expression direct hybridization assay system from Illumina. Finally, the BeadChips were scanned using the Illumina BeadArray Reader. The data was extracted using BeadStudio 3.2 software (Illumina). Data normalization and gene differential expression analyses were conducted using the Rosetta error models available in the Rosetta Resolver® system (version 7, Rosetta Biosoftware, Seattle, WA, USA). This model transforms and processes the data based on modeling with same-versus-same comparisons where the overall number of genes observed to be significant in a ratio plot is below the p value. As individual replicates are combined from intensity profiles to intensity experiments, the errors are also combined so that true differences have lower errors, and in the final ratio plot, this additive effect does tend to yield true positives, especially with additional fold change criteria. Fold changes and p values were generated based on an intensity ratio between control and disease using a conversion pipeline provided by the Rosetta Resolver® system (Weng et al. 2006). Intensity values of individual genes are presented untransformed. A principal component analysis was first carried out to detect low quality arrays. A cluster analysis using a hierarchical algorithm (agglomerative) was conducted next to detect potential outliers within each cohort. Gene lists containing statistically significant ($p \leq 0.05$) differentially regulated genes were generated for each disease in the first instance and compared. Fold change (FC) was then considered and described in more detail hereafter. The comparison of all possible combinations of diseases was considered without prior assumptions. A gene set enrichment analysis (GSEA) was also conducted using Pathway Studio 6.0 (Ariadne Genomics, Rockville, MD, USA) and commonly dysregulated biological processes determined across all six diseases.

Data accession

All microarray data are available online through Gene Expression Omnibus (accession number GSE26927). In total data from 118 samples were deposited. This excludes human brain reference replicated several times across the arrays for inter-chip normalization purposes and two arrays due to technical error (poor hybridization).

Quantification of mRNA expression by RT-qPCR

The two-step real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed using the QuantiTect® reverse transcription kit, the QuantiTect® SYBR Green kit and with QuantiTect® primer assays (Qiagen) as previously described (Durrenberger et al. 2010). Briefly, real-time PCR experiments were performed using the Mx3000P™ real-time PCR system with software version 4.01 (Agilent technologies UK Ltd, Stockport, UK). The QuantiTect® primer assays are listed in Table 2. For each sample, reactions were set up in duplicate with the following cycling protocol, 95 °C for 15 min, 40 cycles with a 3-step program (94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s) and a final melting curve analysis with a ramp from 55 to 95 °C.

We had also established the most stable expressed gene using the coefficient of variance (CV) for all genes across

Table 2 List of primers in alphabetical order

Symbol	Name	Entrez gene ID	Amplicon length	Catalog number
BECN1	Beclin 1, autophagy related	8678	150 bp	QT00004221
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein	241	120 bp	QT00077252
CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	1573	87 bp	QT00027139
ELF1	E74-like factor 1 (ets domain transcription factor)	1997	130 bp	QT00023716
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	2597	119 bp	QT01192646
HLA-DRA	Major histocompatibility complex, class II, DR alpha	3122	101 bp	QT00089383
HLA-DRB4	Major histocompatibility complex, class II, DR beta 4	3126	124 bp	QT00091889
IL13RA1	Interleukin 13 receptor, alpha 1	3597	68 bp	QT00083482
SST	Somatostatin	6750	6,750	QT00004277
TIMP	TIMP metallopeptidase inhibitor 1	7076	115 bp	QT00084168
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	7132	96 bp	QT00216993
TNFRSF14	Tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	8764	116 bp	QT00082432
TYROBP	TYRO protein tyrosine kinase-binding protein	7305	128 bp	QT00077518
XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	7511	112 bp	QT00051471

The sequences of the Qiagen primers are proprietary information of Qiagen and details of the primers can be found at the Qiagen GeneGlobe™ Search Center (www.qiagen.com/GeneGlobe)

all samples. A candidate reference gene (constitutively expressed gene irrespective of disease state) was selected based on rank, function, and lack of involvement in neurodegeneration established by a Pubmed search. *XPNPEP1* [X-prolyl aminopeptidase (aminopeptidase P) 1, soluble] was selected (Durrenberger et al. 2012a). Expression levels of target genes were normalized to the levels of the novel *XPNPEP1* reference gene and calibrated utilizing a standard curve method for quantitation. Some results were duplicated using a more commonly used reference gene (data not shown).

Statistical analysis

The following software packages were used, GraphPad Prism 5.01 (GraphPad Software Inc, La Jolla, CA, USA) and Microsoft® Office Excel® 2007 (Microsoft UK Headquarters, Reading, UK). For a particular gene, gene expression signal intensity data and RT-qPCR relative expression data was divided by the mean of the control group in each respective disease and presented as a ratio (labeled as expression ratio). This procedure scaled the mean gene expression levels of the control group at one for all genes and diseases. The ratio data is presented as mean ± standard error of means (SEM). The Pearson's product-moment correlation test (2-tailed) and linear regression were applied to determine the relationship between two variables. Group difference, other than microarray, was established using an independent t test or student's t test for unequal variance established by F test (2-tailed or 1-tailed whenever appropriate). Fisher's exact test was used as a non-parametric test to compare gender. An F test was used to establish homogeneity of variance. Differences were considered statistically significant if the $p \leq 0.05$.

Results

Quality control assessment

Once the gene expression data was loaded into the Rosetta Resolver® system, the data from each array was first reduced into a three-dimensional space by conducting a principal component analysis (PCA). PCA simplifies complex data sets by reducing the number of variables into a 2–3-dimensional space more suited for visualization (Stekel 2003). This statistical method permits the isolation of arrays that have an abnormal overall intensity signal. Out of the 128 arrays, 2 arrays showed extreme low intensity signal due to poor hybridization. Another array within the HD group was also an outlier which was correlated to a lower RIN (RNA integrity number) value. These three arrays were, therefore, excluded from the normalization and analysis (Fig. 1a). Furthermore, a cluster analysis was conducted for each disease to determine whether control and disease cases clustered within their respective experimental group. Only one outlier was removed that did not cluster within the MS cohort, due to

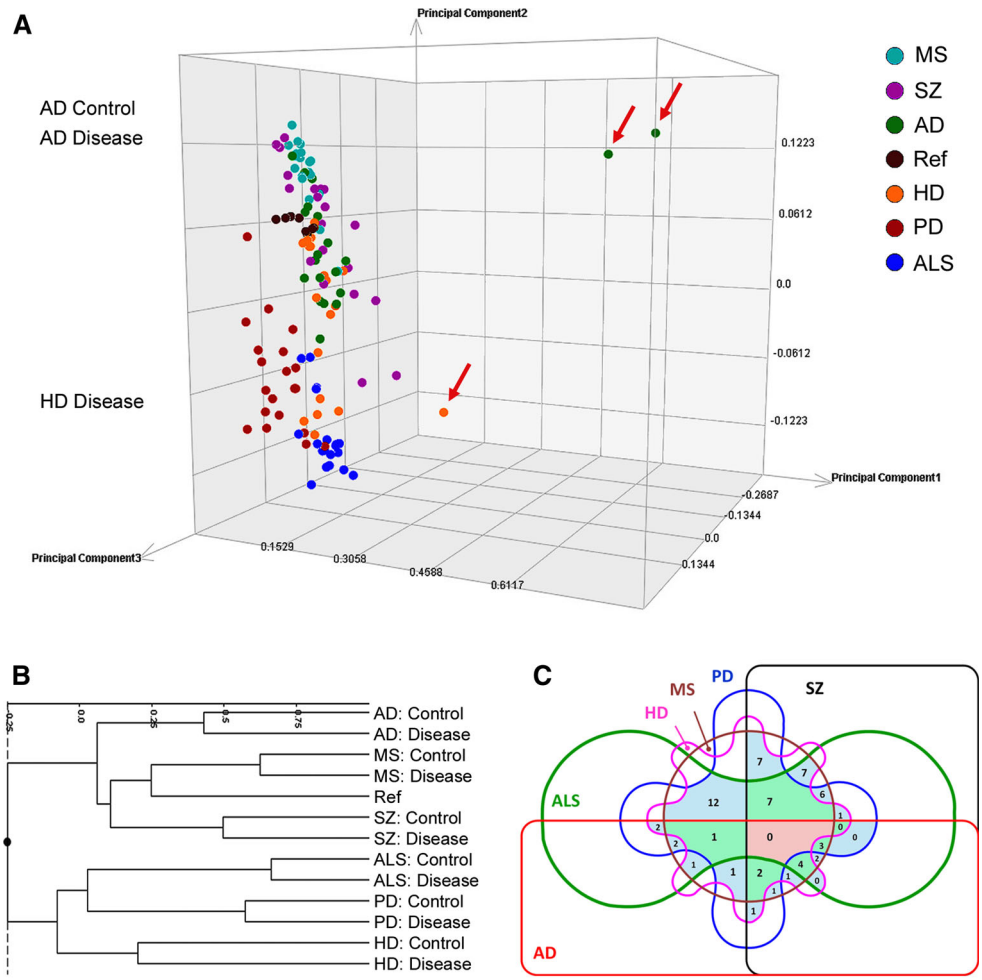

Fig. 1 Principal component, cluster analysis, and gene list comparison. Three low quality arrays were detected with the principal component analysis, which were subsequently removed from the analysis. These arrays showed a reduced signal frequency distribution compared to a normal bell-shaped signal frequency distribution (**a**). The cluster analysis showed that all diseases clustered with their respective controls (**b**). All possible combinations of diseases with respective significant retained dysregulated genes (**c**). In *red* the 6-disease intersect, in green the 5-disease intersects and in *blue* the 4-disease intersects (Fig. 1**c**: reproduced with permission see Wikimedia commons under author name Durrenberger)

molecular signs of hypoxia/ischemia. Finally, when conducting a cluster analysis on all the experimental groups, all disease groups clustered well with their respective controls and segregated as specific brain regions (Fig. 1b).

Lists of differentially expressed genes were generated for each condition at a significance level of $p \leq 0.05$ using the Rosetta error model. Signal intensities below background with a signal detection $p \geq 0.05$ were not considered. Because we opted for an analysis without prior assumptions concerning disease associations or pathways, all possible disease combinations were analyzed in the first instance, although not all are discussed here in detail. We then established an average FC irrespective of its directionality and an average p value for each gene across diseases. A total of 61 genes were retained (Fig. 1c and Online Resource 2). For five diseases, 17 genes with an average FC > 1.4 (range of FC for those 17 genes: 1.41–2.1) and for four diseases, 44 genes with an average FC ≥ 1.6 (range: 1.6–2.82) were retained. Two genes were found to be upregulated in common between all six diseases; Axin-2, part of the Wnt/β catenin signaling pathway and another of unknown function, ZCCHC14 (zinc finger, CCHC domain containing 14), but their average FC was small ($\times 1.25$, $p = 0.012$ and $\times 1.23$, $p = 0.016$; respectively).

To verify the general findings from the microarray data, we replicated expression levels using RT-qPCR for 11 genes in multiple diseases which generated a total of 46 PCR experiments. We selected several receptor and/or effector molecules which could be mapped onto various biological processes or cell activities believed to be relevant in helping us to further our understanding of pathogenetic pathways of neurodegeneration. The following genes were selected (in alphabetical order): Arachidonate 5-lipoxygenase-activating protein (ALOX5AP); cytochrome P450, family 2, subfamily J, polypeptide 2 (CYP2J2), E74-like factor 1 (ELF1); major histocompatibility complex, class II, DR alpha (HLA-DRA); major histocompatibility complex, class II, DR beta 4 (HLA-DRB4); interleukin 13 receptor, alpha 1 (IL13RA1); somatostatin (SST); TIMP metallopeptidase inhibitor 1 (TIMP1); tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A); tumor necrosis factor receptor superfamily, member 14 (TNFRSF14) and TYRO protein tyrosine kinase-binding protein (TYROBP aka DAP12). In

addition to generating a FC and p value (Student's t test) for each individual PCR experiment, we also correlated microarray and the qPCR individual expression data. There was an excellent overall correlation (Online Resource 3). We then compared fold changes generated by both hybridization methods and found a highly significant correlation (xy pairs = 37; $r^2 = 0.91$; $p < 0.0001$; Fig. 2). Finally, to summarize expression level of a gene in NDs, we normalized and collapsed all data (same directionality only) and gave an overall statistical significance (t test; 2-tailed for microarray and 1-tailed for RT-qPCR).

Common genes in neurodegenerative diseases

The functional category most represented for the 61 retained genes was immune response (30 % of the genes, 20/61). The remaining genes were grouped in the following categories: signal transduction (5 genes), angiogenesis (3), nervous system development (3), oxidation reduction (3), apoptosis (3), and synaptic transmission (2). In addition to the direct gene list comparison, we also conducted a GSEA on each entire dataset (at $p \leq 0.05$) to isolate gene sets that share similar biological functions (Subramanian et al. 2007). The biological processes in common for all six diseases were as follows (in alphabetical order): antigen processing and presentation, cell adhesion, inflammatory response, regulation of cell proliferation, pattern specification process (referring to a developmental process),

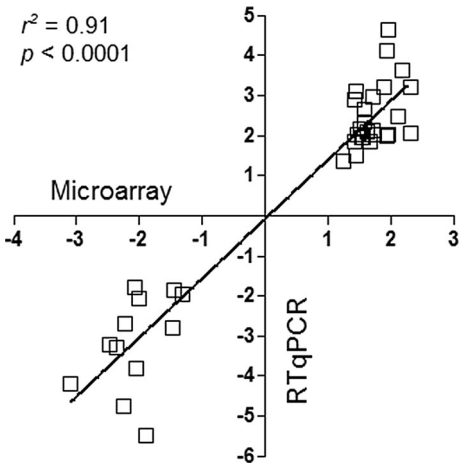

Fig. 2 Comparison of gene expression levels from the microarray and RT-qPCR. Forty-six experiments were conducted comparing expression levels of 11 genes across several diseases. Fold changes from both experiments were compared but only when group differences were statistically significant using both methods. Results are plotted as fold changes and fold differences of relative gene expression between disease and control. We also correlated individual sample expression levels from both methodologies for each experiment. There was a significant correlation ($r^2 = 0.9115$; $p < 0.0001$) overall on the fold changes from all experiments

response to drug and nutrient and skeletal development. This inter-disease comparison shows strong evidence of the involvement of common effector innate immune response components in the disease pathogenesis and/or compensatory mechanisms. Since pathway analysis was unable to find direct links between the 61 genes, the data was interpreted in the context of a neuroinflammatory response. Damage or stress to cells results in a glial and/or neuronal response that leads to pro-inflammatory cytokine release and consequently a state of chronic inflammation (Jellinger 2010).

Cellular stress and neurodegeneration

Several gene expression changes were indicative of cellular stress, including SLC7A9 [Solute carrier family 7 (cationic amino acid transporter, y + system)], which was dysregulated in ALS, HD, MS, PD, and schizophrenia. SLC7A9 is a glycoprotein-associated amino acid transporter showing high-affinity for L-cystine (Verrey et al. 2004), which is essential in the synthesis of antioxidant glutathione released in response to oxidative stress (Aoyama et al. 2008). In addition, the heat shock protein DNAJB6 (DnaJ (Hsp40) homolog, subfamily B, member 6) was upregulated in HD, MS, and PD. Indications of common apoptotic pathways were highlighted by changes of B-cell CLL/lymphoma 2 (BCL2), known to be involved in the regulation of apoptosis (Ow et al. 2008) as well as cell cycle control (Zinkel et al. 2006). BCL2 was upregulated in AD, HD, MS, and SZ. Very few neuronal and oligodendrocyte-specific genes were found to be dysregulated in common. One of the exceptions was somatostatin (SST), a peptide hormone modulating neurotransmission, which was downregulated in ALS, HD, MS, and SZ (Fig. 3a, b).

Glial reactivity

Microglia and astrocytes show a rapid response to any disturbance in the CNS microenvironment and glial reactivity results in an increased expression of specific cell surface receptors, such as MHC (major histocompatibility complex) class II on microglia, and in the release of growth factors and cytokines, which may be protective acutely but, if not resolved, may result in impaired neuronal function. The MHC class II receptors, HLA-DRA (Fig. 3c, d) and HLA-DPA1 were significantly upregulated in neurodegenerative disorders and downregulated in schizophrenia. The MHC class II receptor, HLA-DRB4 was upregulated in AD, MS, PD (Fig. 3e, f), and again downregulated in schizophrenia. Moreover, other genes known to be restricted to cells of the monocytic lineage (microglia and/or perivascular macrophages), or known to be directly involved with the innate immune response, have also been

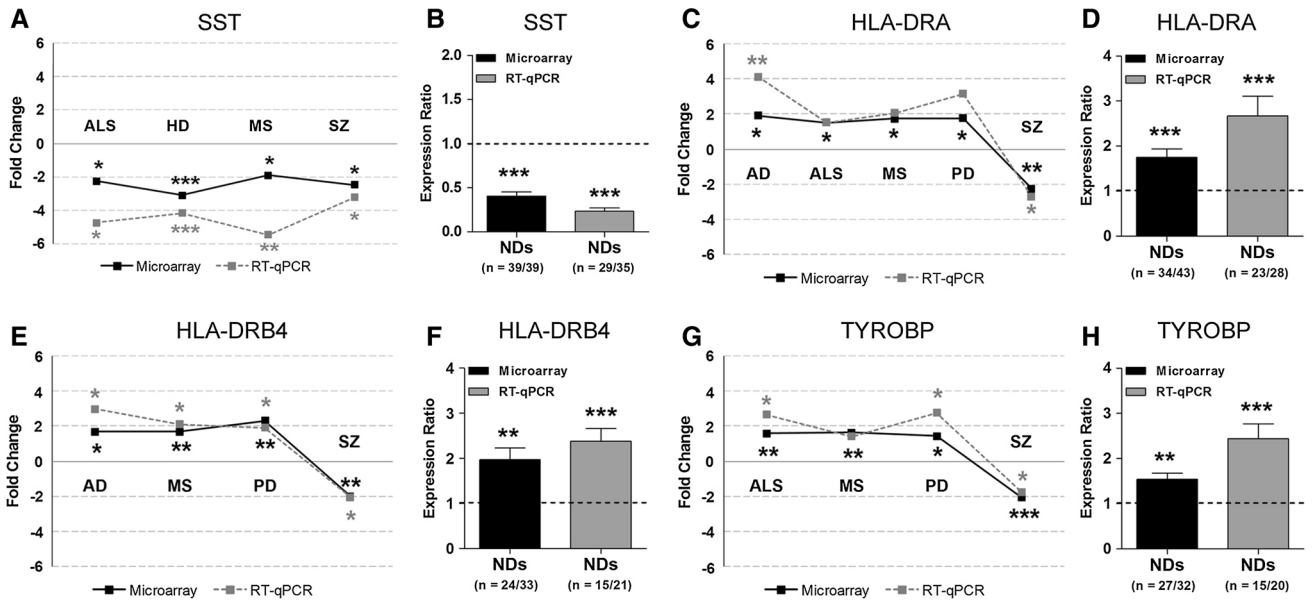

Fig. 3 Expression levels of SST, HLA-DRA, HLA-DRB4, and TYROBP from microarray and RT-qPCR experiment. Expression levels of HLA-DRA, HLA-DRB4, and TYROBP were significantly increased in neurodegenerative disorders while SST was significantly downregulated $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

highlighted in our dataset. These include the triggering receptor expressed on myeloid cells (TREM2), TYRO protein tyrosine kinase protein (TYROBP/DAP12; Fig. 4g, h), CD74 (CD74 molecule, MHC class II invariant chain), carboxypeptidase vitellogenic-like (CPVL), GRAM domain containing 1C (GRAMD1C), annexin A1 (ANXA1) and RFX4 v3 (RFX4 regulatory factor X4, influences HLA class II expression). Finally, astrocytes are known to release transforming growth factor β in response to neuronal injury. Transforming growth factor β receptor 2 (TGFBR2) was upregulated in AD, HD, MS, and PD.

Other inflammation-related genes

A number of dysregulated genes were indicative of ongoing inflammation or modulation of the adaptive immune system. For example, IL13RA1 (Interleukin 13 receptor, alpha 1) was significantly upregulated in ALS, HD, MS, PD, and downregulated in schizophrenia (Fig. 4a, b). Others include tumor necrosis factor receptor superfamily member 1A (TNFRSF1A; Fig. 4c, d) that binds TNF, the TNFRSF14 receptor (alias herpesvirus entry mediator; Fig. 4e, f), cytochrome b-245, alpha polypeptide (CYBA), parvin gamma (PARVG) and E74-like factor 1 (ELF1; Fig. 4g, h). Several members of the arachidonic acid pathway were also found to be dysregulated such as ALOX5AP (Fig. 5a, b) and CYP2J2 (Fig. 5c, d). Finally, our data also showed indications of common cerebral endothelial tight junction and adhesion molecule abnormalities across several diseases, such as upregulation of ADAM metallopeptidase with thrombospondin type 1 motif 9 (ADAMTS9) and of TIMP metallopeptidase inhibitor 1 (TIMP1; Fig. 5e, f).

Discussion

Our extensive single microarray platform analysis of genome-wide changes in human brain tissue from AD, ALS, HD, MS, PD, and schizophrenia allowed us to overcome some of the challenges previously encountered by transcriptomic approaches, such as the use of different microarray platforms and/or different analytical software tools, and used an unbiased approach to reveal shared single genes across different neurodegenerative conditions. Our correlative analysis of the microarray and qPCR results shows that the Illumina BeadChip platform is a very reproducible and sensitive system for studying gene expression changes in human post-mortem tissues. The direct comparison of the lists of dysregulated genes showed that of the 61 genes found in common between at least four diseases, 20 had an immune/inflammation-related function. This comparison between six major CNS disease states showed strong indications of common changes in the regulation of effector immune responses and CNS tissue inflammation in neurodegenerative diseases that may contribute toward or exacerbate the fate of the different susceptible neuronal populations, but at the same time also highlighted the diversity of molecular mechanisms.

Neurodegenerative diseases, although clinically characterized as distinct entities, exhibit common neuropathological changes (Armstrong et al. 2005). By selecting well-

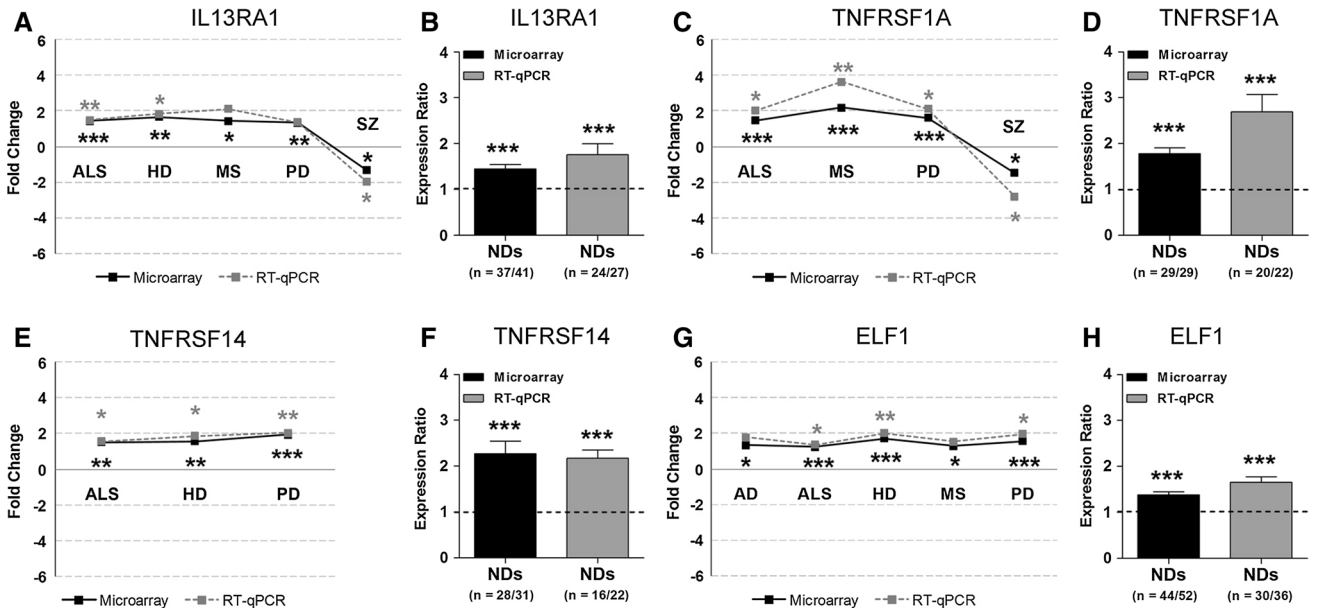

Fig. 4 Expression levels of IL13RA1, TNFRSF1A, TNFRSF14, and ELF1 from microarray and RT-qPCR experiment. Expression levels of all genes were significantly increased in neurodegenerative disorders $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

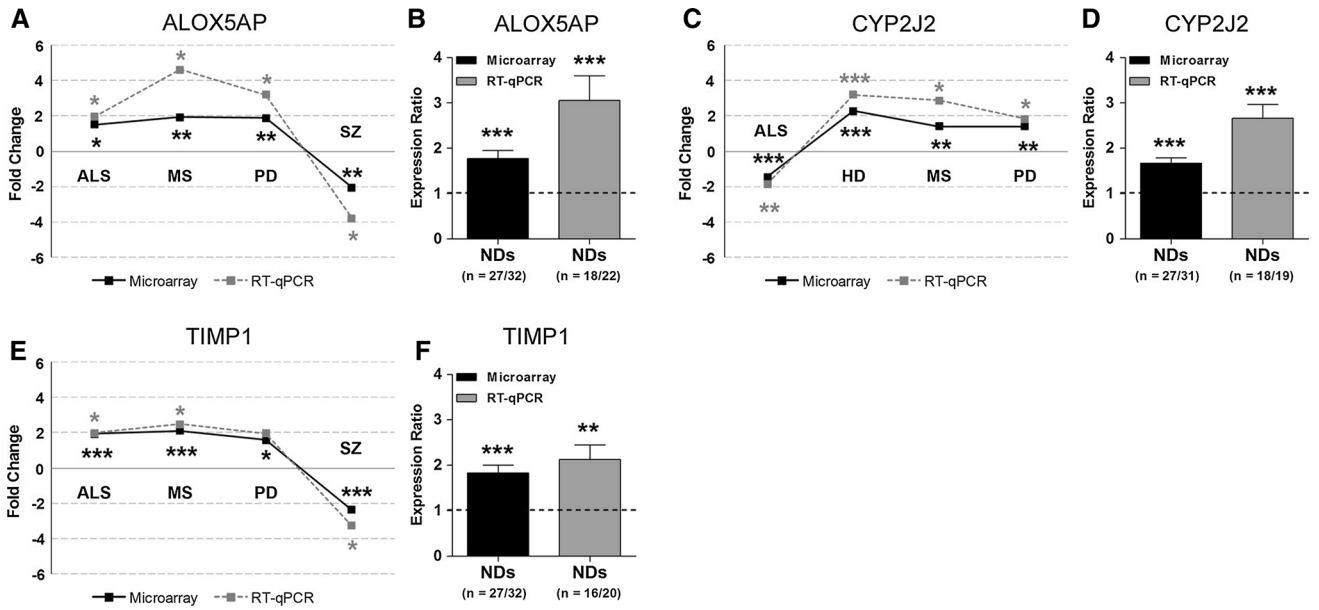

Fig. 5 Expression levels of ALOX5AP, CYP2J2 and TIMP1 from microarray and RT-qPCR experiment. Expression levels of all genes were significantly increased in neurodegenerative disorders $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

characterized and optimally preserved post-mortem brains at a stage when ongoing neuronal loss was occurring, we were able to demonstrate that, despite high number of significant dysregulated genes in individual diseases (Durrenberger et al. 2012b; Schmitt et al. 2011, 2012), only a few single specific genes could be found in common. This would suggest that, despite all our standardization efforts, a direct gene comparison approach may not be the most yielding method in determining commonalities at the molecular level across NDs or that common molecular mechanisms do not exist. This only reinforces the pathological specificity of the disease microenvironment in the conditions studied herein, and most importantly, that at a molecular level the common features, such as microglial activation, can occur via diverse mechanisms that are disease specific and may result in different outcomes. Hence,

there is a need to re-address the exact nature of those features in each disease. Nevertheless, the few genes found in common reflected both the degenerative process and the ongoing attempts of the brain to protect against or cope with neuronal cell death. As a result, the main and most interesting finding was that of changes to the innate immune system involvement suggesting immunoregulatory and immunomodulatory mechanisms. We have interpreted the outcome of this study within the current framework of neuroinflammation since no direct link was found between the 61 genes. It is well accepted that glial activity in response to damaged or stressed cells can lead to the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and consequently leads to a state of chronic inflammation (Jellinger 2010). As a consequence of an abnormal sustained inflammation, the brain's homeostasis is jeopardized and the allostatic load increased (Saavedra 2011). The (innate) immune system is central to maintain the brain's homeostasis with the resolution of inflammation as a key element (Chen and Nunez 2010) and remains, for the future, one of the main targets for potential therapeutic interventions (Amor and Woodroofe 2014).

Cellular stress and death/survival

Our dataset highlighted apoptosis and a general cellular stress response in the neurodegenerative and/or neuroprotective process. Neuronal cell death occurring by apoptosis has been reported in several NDs (Mattson 2006), including MS (Magliozzi et al. 2010). Although increased levels of TNF mRNA were not detected by our study, elevated soluble TNF expression is a confirmed hallmark of acute and chronic neuroinflammation and has been observed in a number of neurodegenerative diseases (Allan and Rothwell 2001; McCoy and Tansey 2008; Gardner et al. 2013). Our observation of an upregulation of the TNFRSF1A gene encoding the TNF receptor 1A in multiple conditions suggests the involvement of changes to TNF signaling pathways that may change the balance between apoptosis and survival and warrants further investigation. The chronic expression of high TNF levels has been shown to lead to progressive neuronal loss in models of PD (Chertoff et al. 2011) and an increased expression of TNFRSF1A would be in keeping with this pathogenetic mechanism.

The cellular response to stress in NDs was highlighted by the upregulation of heat shock protein 40 (DNAJB6) expression. The paucity of changes in gene expression related to oxidative stress is probably due to the fact that the majority of changes are post-translational (Martinez et al. 2010). However, endoplasmic reticulum stress may trigger cell stress responses involving aberrant protein folding, thus explaining the response of chaperones. Overexpression of Hsp70, Hsp40, and Hsp27 has demonstrated the protective effects of heat shock proteins (HSPs) in several animal models of neurodegenerative diseases (Muchowski and Wacker 2005). Increased mRNA levels for HSPs in PD were also reported by another study using a different microarray platform (Durrenberger et al. 2009). This study showed a strong expression of DnaJB6 expressed by astrocytes, which could reflect a protective reaction, so reducing the neuronal release of toxic alpha-synuclein and supporting the idea that the astrocyte response might limit the neurodegenerative process.

Neuroprotective effects were further shown with increased levels of transforming growth factor-beta (TGF-β). Transforming growth factor, beta receptor II (TGFBR2) was upregulated in AD, HD, MS, and PD and increased levels are in accord with previous evidence that reported increased TGFBR2 expression in various neuronal populations, activated astrocytes, and ramified microglia (Pratt and McPherson 1997). TGF-β1 stabilises Ca^{2+} homeostasis via the N-methyl-D-aspartate receptors and in vivo studies have demonstrated that in models of cerebral ischemia administration of TGF-β reduced brain lesions (Vivien and Ali 2006). In addition, TGF-β has survival promoting effects on dopaminergic neurones, both in vitro and in vivo (Roussa et al. 2009). Receptor expression is thought to be directly increased as a consequence of neuronal stress and is considered to have a neuroprotective effect and as a potential therapeutic target (Vivien and Ali 2006).

Microglia and neuroinflammation

It is generally acknowledged that microglial activation is characteristic of all disorders of the CNS, although their dual role in neuroprotection and neurodegeneration is still hotly debated (Amor and Woodroofe 2014; Ransohoff and Cardona 2010). Therefore, it is not surprising that a number of genes associated with various effector functions of microglia (Mosher and Wyss-Coray 2014) were highlighted by our study. It is likely that this reflects the chronic activation state of microglia. Activation of innate immunity in the CNS is usually characterized by increased MHC complex class II expression in response to extracellular apoptotic material and many genes involved in this process (HLA-DRA, HLAD-PA1, HLA-DRB4) were found to be upregulated in our study. MHC class II is also a marker of the "primed" microglia phenotype reported in AD (Parachikova et al. 2007) and PD (Imamura et al. 2003). In addition, genes associated with antigen presentation (CD37, CD74, and RFX4 v3), or found specifically on cells of myeloid lineage (CPVL and TREM2), were upregulated in all the conditions studied, expect for schizophrenia where they were mainly downregulated. The finding that a significant number of genes involved in immune system

function and inflammatory processes were downregulated in schizophrenia is intriguing and clearly highlights a distinction between this disorder and the neurodegenerative diseases. This finding has been described and discussed in detail elsewhere (Schmitt et al. 2011) and is indicative of abnormal or failing immune regulation in schizophrenia, which could have a detrimental effect on the maintenance of the synaptic network and consequently lead to abnormal cognitive functions.

Upregulation of ANXA1, observed in AD, ALS, and HD, is also consistent with a microglial protective response (Solito et al. 2008). Molecules involved in antigen presentation are thought to play crucial roles in mediating microgliosis and consequently neurodegeneration (Gao and Hong 2008). It is increasingly suggested that chronic neuroinflammation involving predominantly microglial activation could be responsible for progressive neuronal loss in neurodegenerative conditions through complex interactions between oxidative stress, iron metabolism, cytokine toxicity, and mitochondrial dysfunction (Urrutia et al. 2014). This was further confirmed by increased TREM2 expression levels in ALS, AD, PD, and MS. New reports suggest that variants of the TREM2 gene may cause increased susceptibility to late onset AD (Hickman and El Khoury 2014). However, TREM2 may also be involved in a protective response as indicated by reduction in TNF and nitric oxide synthase-2 production when over-expressed in microglia (Takahashi et al. 2005). TREM2 associates with DAP12, an intracellular signaling subunit, to either activate or inhibit the immune response (Lanier 2009) and the TREM2/DAP12 complex is strongly expressed by microglia and to a certain extent by neurones, but not by other glia (Sessa et al. 2004). Blockade of TREM2 during the effector phase of experimental autoimmune encephalomyelitis (EAE) resulted in disease exacerbation with more diffuse CNS inflammatory infiltrates and demyelination in the brain parenchyma (Piccio et al. 2007), again suggesting a protective role for this molecule in microglia. We found that expression levels of DAP12 (ALS, MS, PD, and SZ) showed the strongest correlation with TREM2. The role of TREM1/DAP12 in systemic inflammation has been established, but very little is currently known about the TREM2/DAP12 complex in the CNS.

Interleukin-13 (IL-13) is one of the major fibrogenic cytokines prominent at sites of Th2 inflammation and a potent stimulator of eosinophil-, lymphocyte-, and macrophage-rich inflammation and parenchymal remodeling (Ma et al. 2006; Martinez et al. 2009). IL-13 receptor $\alpha 1$ binding initiates the activation (or shift) of mononuclear phagocytes into the M2 phenotype, which are suggested to play a role in the resolution of inflammation by producing anti-inflammatory mediators. By production of profibrotic factors such as fibronectin, matrix metalloproteinases (MMPs), IL-1b and TGF-β, M2 macrophages are associated with tissue repair (Fairweather and Cihakova 2009). Thus, the upregulation of IL13RA1 in ALS, HD, MS, and PD suggests a possible shift toward M2-mediated tissue repair functions. Moreover, ELF1 was upregulated in all of the neurodegenerative conditions and has been shown to enhance CD68 activity in vitro (O'Reilly et al. 2003). Although it is believed that crosstalk between the innate and adaptive immune system is kept to a minimum due a high threshold for lymphoid activity in the CNS, new evidence suggests that the CNS interacts with the adaptive immune system and several CNS-specific mechanisms of local T-cell response regulation have been proposed (Tian et al. 2009). Regulating the cross-talk between the adaptive and innate immune systems could prove beneficial in the long-term treatment of NDs and there is accumulating evidence to suggest that the BBB is altered in many CNS disorders (Zlokovic 2008). It is increasingly realized that vascular changes, systemic cytotoxic mediators and cells of the adaptive immune system may play a major role in neurodegeneration and several routes of entry have been put forward (Ransohoff et al. 2003). Over-expression of TIMP-1 has been associated with attempts to compensate for BBB leakages and has been suggested to be neuroprotective (Fujimoto et al. 2008), which is in keeping with our observation that TIMP-1 gene expression was upregulated in ALS, HD, and MS. Although vascular dysfunction is a known contributor to neurodegeneration in dementias, the exact involvement of endothelial cell changes in disease progression remains to be fully understood and further studies are required to determine the functional significance of these changes.

Neuron and glial specific genes

There were few indications of common abnormalities in neuron-specific gene expression across the various neuronal populations investigated, with the exception of the neuropeptide somatostatin (SST) which was downregulated in ALS, HD, MS, and schizophrenia. Previous reports of somatostatin loss and its receptors has been reported in AD (Burgos-Ramos et al. 2008), HD (Timmers et al. 1996), PD (Agnati et al. 2003) and schizophrenia (De Wied and Sigling 2002) and is suggested to be related to cognitive impairment. This absence of common neuronal pathways may not be unexpected since different anatomical brain areas, disease stages, disease heterogeneity, and a variety of neuronal populations were investigated. Downregulation of dopamine-related genes was observed but remained specific to HD and PD (data not shown). Furthermore, neurofilament heavy polypeptide was downregulated in AD, HD, and PD (average fold change x-1.72, $p < 0.03$) and neurofilament medium polypeptide in HD, MS, and PD

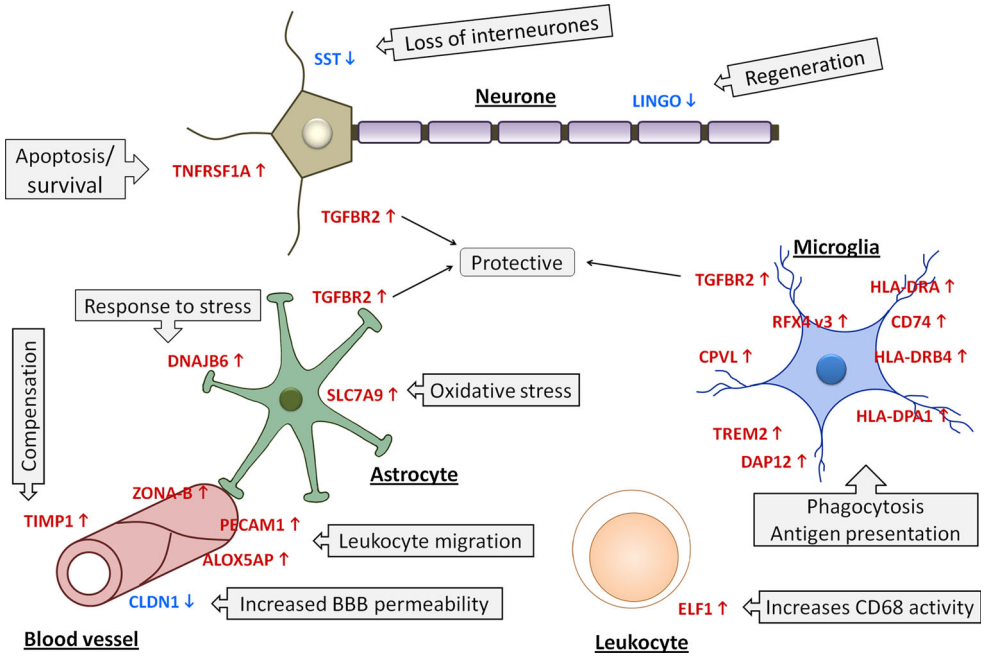

Fig. 6 Schematic representation of molecular mechanisms highlighted by the dataset. Common genes, associated with a known cell phenotype and biological process, are represented on this drawing to highlight the some of the pathogenetic mechanisms. The few genes found in common suggest cellular efforts of common neuronal homeostatic and survival activity and of immunoregulatory and immunomodulatory mechanisms including the resolution of inflammation which is generally supportive of the neuroinflammatory hypothesis in neurodegenerative disorders

(average fold change x-2.18, $p < 0.01$). Also, synaptic genes, interneurone markers, a large number of K+ and Na+ channels were downregulated in several diseases (data not shown). This is in keeping with a general loss of neurones, which may occur to a different extent in all diseases. Our dataset would suggest that each neuronal population is distinct and unique and that this distinctiveness is extended to their microenvironment with little common homeostatic and supportive mechanisms from neighboring cells. Consequently, some of the genes revealed by this dataset may be common in neurodegenerative diseases but appear indubitably to lead to specific neuronal changes.

Conclusion

In conclusion, several dysregulated genes were found in common across the major CNS diseases under study suggesting changes to a number of biological processes (Fig. 6). This is the first time that a direct gene comparison has been applied to major neurodegenerative diseases in a single study in a reproducible way. Our initial analysis of these extensive datasets reveals that the molecular basis of the shared features in neurodegenerative diseases may not be as common as initially thought. This study reinforces the uniqueness of the each disease microenvironment. Nevertheless, the few genes found in common suggest cellular efforts of common neuronal homeostatic and survival activity and of immunoregulatory and immunomodulatory mechanisms including the resolution of inflammation which is generally supportive of the neuroinflammatory hypothesis in neurodegenerative disorders. Unraveling the detailed nature of these molecular changes will be key to understand the complex pathogenetic mechanisms involved in these chronic conditions and their potential reversal.

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Conflict of interest None declared.

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