

Gene expression profiling of substantia nigra dopamine neurons: further insights into Parkinson's disease pathology

Filip Simunovic,¹ Ming Yi,² Yulei Wang,³ Laurel Macey,¹ Lauren T. Brown,¹ Anna M. Krichevsky,⁴ Susan L. Andersen,⁵ Robert M. Stephens,² Francine M. Benes⁶ and Kai C. Sonntag¹

1 Department of Psychiatry, McLean Hospital, Harvard Medical School, Belmont, MA 02478, USA

2 Advanced Biomedical Computing Center, Advanced Technology Program, SAIC-Frederick, Inc., NCI-Frederick, Frederick, MD 21702, USA

3 Applied Biosystems, Foster City, CA 94404, USA

4 Department of Neurology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

5 Laboratory for Developmental Neuropsychopharmacology, McLean Hospital, Harvard Medical School, Belmont, MA 02478, USA

6 Program in Neuroscience and Department of Psychiatry, McLean Hospital, Harvard Medical School, Belmont, MA 02478, USA

Correspondence to: Kai-Christian Sonntag, MD, PhD,
Department of Psychiatry, McLean Hospital,
Harvard Medical School, MRC 223,
McLean Hospital, 115 Mill Street,
Belmont, MA 02478, USA
E-mail: ksonntag@mclean.harvard.edu

Parkinson's disease is caused by a progressive loss of the midbrain dopamine (DA) neurons in the substantia nigra pars compacta. Although the main cause of Parkinson's disease remains unknown, there is increasing evidence that it is a complex disorder caused by a combination of genetic and environmental factors, which affect key signalling pathways in substantia nigra DA neurons. Insights into pathogenesis of Parkinson's disease stem from *in vitro* and *in vivo* models and from postmortem analyses. Recent technological developments have added a new dimension to this research by determining gene expression profiles using high throughput microarray assays. However, many of the studies reported to date were based on whole midbrain dissections, which included cells other than DA neurons. Here, we have used laser microdissection to isolate single DA neurons from the substantia nigra pars compacta of controls and subjects with idiopathic Parkinson's disease matched for age and postmortem interval followed by microarrays to analyse gene expression profiling. Our data confirm a dysregulation of several functional groups of genes involved in the Parkinson's disease pathogenesis. In particular, we found prominent down-regulation of members of the PARK gene family and dysregulation of multiple genes associated with programmed cell death and survival. In addition, genes for neurotransmitter and ion channel receptors were also deregulated, supporting the view that alterations in electrical activity might influence DA neuron function. Our data provide a 'molecular fingerprint identity' of late-stage Parkinson's disease DA neurons that will advance our understanding of the molecular pathology of this disease.

Keywords: Parkinson's disease; microarray; laser microdissection; pathogenesis; dopamine

Abbreviations: DA = dopamine; LMD = laser microdissection; PCD = programmed cell death; PMI = postmortem interval; UPS = ubiquitin-proteasome system

Introduction

Parkinson's disease is a neurodegenerative disorder caused by a progressive deterioration of midbrain dopamine (DA) neurons in the substantia nigra pars compacta (SNc). The death of DA cells is associated with tremor and rigidity and results in a gradual dysfunction of the extrapyramidal motor system. The disease affects about 2–3% of individuals over the age of 65 years and there is evidence that its prevalence is higher in the male population (Cantuti-Castelvetri *et al.*, 2007). There is currently no cure for Parkinson's disease and the underlying pathogenesis of the disease is still unknown. Two forms of Parkinson's disease are recognized: a 'familial' or early-onset Parkinson's disease (<10% of all patients) and an 'idiopathic' or late-onset Parkinson's disease (>85% of all cases) that does not appear to exhibit heritability. Overall, the pathology of Parkinson's disease is complex and is most likely a 'consequence of an unspecified combination of genetic and environmental factors, which induce a common pathogenic cascade of molecular events' (Maguire-Zeiss and Federoff, 2003; Miller and Federoff, 2005).

Since the first description of this syndrome in 1817 by James Parkinson, Parkinson's disease has been the subject of intense investigation to understand its pathophysiology and to develop therapeutic interventions. So far, pharmacological and surgical therapies are available and can alleviate some of the symptoms, but these interventions are associated with serious side effects and generally lose efficacy over time (Benabid, 2007; Schapira, 2007). Although research has progressed, one of the main hurdles for the development of therapeutic or preventative measures is the still limited understanding of the underlying pathophysiology of Parkinson's disease and the lack of reliable biomarkers. To a large extent, biomedical research on Parkinson's disease focuses on *in vitro* and *in vivo* disease models, as well as studies of post-mortem brain. Based on the availability of more sophisticated technologies, the latter has become more prominent over the past years and has revealed novel insights in the pathogenesis of Parkinson's disease. For example, several studies have used microarray technologies on the substantia nigra of normal control and Parkinson's disease patients to assess differential gene expression profiles; data from these studies have helped to further delineate some disease-associated pathways (Grunblatt *et al.*, 2004; Hauser *et al.*, 2005; Zhang *et al.*, 2005; Duke *et al.*, 2006; Miller *et al.*, 2006; Moran *et al.*, 2006, 2007; Moran and Graeber, 2008). However, the array results in these studies did not entirely represent the DA neuronal profile, since large amounts of other cell populations were also included in the dissected tissue. The introduction of laser microdissection (LMD) has further refined this approach and was essential to the demonstration of a broad gender-linked difference in the gene expression profile of human substantia nigra DA neurons (Cantuti-Castelvetri *et al.*, 2007).

In the current study, we used LMD (Benes *et al.*, 2007) to isolate DA neurons from the substantia nigra of nine normal and 10 idiopathic Parkinson's disease patients. Using microarray-based gene expression profiling, we have analysed our data based on cluster analyses of biological functions and cellular pathways relevant to Parkinson's disease pathology and have compared the results to the published expression profiles. Our data confirm the

involvement of several known molecular regulatory pathways in the pathogenesis of Parkinson's disease: these include oxidative stress-induced cell responses and dysfunction of the mitochondrial and ubiquitin-proteasome system (UPS). In particular, we found clusters of differentially expressed genes that appear to be involved in extrinsic and intrinsic signalling events in programmed cell death (PCD), as well as a prominent down-regulation of multiple members of the PARK gene family, which are associated with familial forms of Parkinson's disease. In addition, we have also noted changes in the expression of neurotransmitter and ion channel genes that suggest alterations in synaptic activity; the latter have been implicated in the modulation of survival and/or degeneration of DA neurons.

Materials and Methods

Subjects and affymetrix-based microarrays

All affymetrix-based microarrays and data about subjects are publicized at the National Brain Databank and were deposited by Dr. Francine Benes (http://national_databank.mclean.harvard.edu/brainbank). Material collection, preparation and data generation were according to previously published protocols (Benes *et al.*, 2007). Briefly, frozen tissue blocks containing SNc from control subjects and patients with idiopathic Parkinson's disease matched for age and post-mortem interval (PMI) were cut using a Microm HM 560 CryoStar cryostat (8 µm), mounted on LEICA Frame Slides with a PET-membrane (1.4 µm) and placed on a LEICA AS LMD apparatus. Since DA neurons contain neuromelanin, they could easily be visualized and collected using laser-based microdissection. Each vial into which the laser-dissected specimens fell by gravity contained a small volume of a lysis/denaturing solution to inhibit RNase activity. An average of 300 or 700 DA neurons were collected from control subjects or Parkinson's disease patient's brains, respectively. RNA extraction was undertaken with a Qiagen RNeasy Micro Kit (Qiagen, Valencia, CA), and quality was assessed using an Agilent 2100 bioanalyser (Agilent Technologies, Palo Alto, CA). Following the manufacturer's instructions, three rounds of linear amplification of the target was carried out using the MessageAmp aRNA Amplification kit (Ambion, Austin, TX). The use of three rounds of amplification could induce degradation of RNA and potentially bias the microarray data; however, all the samples from both groups were processed in an identical fashion, making it unlikely that such bias occurred in one group to a greater degree than another. Subsequently, target labeling was performed with the Message-AMP Biotin Enhanced Kit (Ambion). Fifteen micrograms of biotinylated target RNA was fragmented and individually hybridized to the HU-133A arrays (Affymetrix, Santa Clara, CA). The microarrays were then stained with two rounds of streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and one round of biotinylated antistreptavidin antibody (Vector Laboratories, Burlingame, CA), scanned twice, and visually inspected for evidence of artefacts.

In addition to their demographic factors, the cases included in this study (Table 1) were chosen on the basis of their RNA quality using tissue pH, the 18S/28S ratio, and the Percent Present Calls for each case as described elsewhere (Luzzi *et al.*, 2003; Benes *et al.*, 2007).

Table 1

Panel A. Statistics of cases used for LMD and mRNA arrays					
Case ID	Assay ID	Age	Primary diagnosis	Gender	PMI
C1	1020	73	Control	M	20.53
C2	1022	89	Control	M	7.4
C3	1024	79	Control	M	20.92
C4	1147	78	Control	M	21.75
C5	1150	75	Control	M	20.12
C6	1151	68	Control	M	16.58
C7	1152	72	Control	F	18.25
C8	1156	69	Control	F	25.15
C9	1157	74	Control	F	12.17
PD1	1143	77	PD	M	10.33
PD2	1144	81	PD	F	17
PD3	1145	79	PD	M	23.42
PD4	1146	72	PD	M	26.25
PD5	1148	73	PD	M	18
PD6	1149	83	PD	M	21.25
PD7	1153	77	PD	M	22.67
PD8	1154	84	PD	F	6.42
PD9	1155	77	PD	M	26.25
PD10	1158	81	PD	F	26.75
Group	Average age	Average PMI	Average age of neurological onset		Average age of psychological onset
Control	75.22	18	0		0
PD	78.4	19.83	65.8		69.25
Panel B. Statistics of cases used for LMD and mRNA arrays by qRT-PCR					
Case ID	Assay ID	Age	Primary diagnosis	Gender	PMI
C3	1024	79	Control	M	20.92
C10		72	Control	M	18.25
C11		71	Control	M	23.40
PD3	1145	79	PD	M	23.42
PD4	1146	72	PD	M	26.25
PD11		68	PD	M	13.92

PD=Parkinson's disease.

Data normalization and analysis

All mRNA chips were normalized using the RMA, or MAS5 procedure in R packages from Bioconductor (www.bioconductor.org), or using GCRMA in Partek Genomic Suite (www.partek.com). For each contrast of classes, probesets were filtered based on the detection calls derived from MAS5 procedure according to the majority rule (for each probeset, in at least one of the classes in contrast it shall have majority of their detection calls as 'P' (present) in the samples of this class in order to be retained in the filtered probeset lists). The data from either RMA or MAS normalization for those filtered probes were subjected to SAM procedure (Tusher *et al.*, 2001) to determine the significant gene lists based on intended false discovery rates (FDR). Student *t*-tests were then used to filter significant gene lists. Alternatively, two- or three-way ANOVA models were used to derive the differentiated genes from different contrasts of different treatment and phenotypes using the Partek Genomic Suite.

The enrichment analysis and pathway-level comparative analysis were performed using the in-house software WPS [(Yi *et al.*, 2006); Yi and Stephens, unpublished results]. Briefly, Fisher's exact test was performed based on 2×2 contingency tables, to determine whether a gene is in a given list and whether it is associated with a pathway (gene set, term). One-sided Fisher's exact test was used to measure whether a particular Biocarta pathway (www.biocarta.com), GSEA gene set term (www.broad.mit.edu/gsea/) or a GO term (www.geneontology.org/) were enriched in a given gene list. The terms were ranked based on their Fisher's exact test *P*-values with the most enriched term listed at the top. To compare biological themes at the pathway, gene set and GO term level across multiple gene lists of different contrasts, these gene lists were also subjected to a pathway-level pattern extraction pipeline (Yi and Stephens, unpublished results). Briefly, after batch computation of Fisher's exact test for the gene lists, the log-transformed *P*-values were retrieved and combined into an enrichment score matrix for cluster analysis or pathway pattern extraction. The terms (pathways, or GO terms) of selected clusters with interests were further used to retrieve the

associated genes from the original gene list. Pathways of interest were displayed along with the data in the WPS program.

The data were also analysed in Partek Genomics Suite to determine the segregation of individual samples and possible differences among control subjects and Parkinson's disease patients (Supplementary Fig. 1S). Although there was a 'batch effect' observed between samples from three different dates of microarray assays (Supplementary Fig. 1SA), this could be compensated by using three-way ANOVA (Supplementary Fig. 1SB). These results demonstrated that all individual samples from normal subjects and Parkinson's disease patients clustered and that there was a clear segregation between normal and disease-association attesting for high consistency and reproducibility of the data.

TaqMan[®] real-time PCR assay validation

Expression of 14 genes (listed below) was measured in three normal control and three Parkinson's disease samples (Table 1) by real-time PCR using TaqMan[®] Gene Expression Assays and the 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA). A total of 250–600 DA neurons were captured from each sample and total RNA isolated using the *mirVANA*[™] miRNA Isolation Kit (Ambion). cDNAs were generated in a 25 µl reverse transcription reaction with 60 ng of total RNA from each sample using the High Capacity cDNA Archive Kit and protocol (Applied Biosystems, PN 4322169). The resulting cDNA was subjected to a 10-cycle PCR amplification followed by real-time PCR reaction using the manufacturer's TaqMan[®] PreAmp Master Mix Kit Protocol (Applied Biosystems, PN 4366127). The 10-cycle pre-amplification protocol has been shown to have 100% efficiency and introduced no bias in fold change determination in a previous study (Li *et al.*, 2008). Four replicates per sample were assayed for each gene in a 384-well format plate. For data normalization across samples, GUSB was used as endogenous control gene. Normalization of Ct values of each gene and determination of fold differences gene expression Parkinson's disease versus control was calculated according to the $2^{-\Delta\Delta Ct}$ method by Livak and Schmittgen (2001; Schmittgen and Livak, 2008). The following genes were analysed:

Gene symbol	Alias	TaqMan assay ID
RAP1GAP	PARK10	Hs00182299_m1
UCHL1	PARK5	Hs00188233_m1
RIMS3	PARK10	Hs00207275_m1
ATP13A2	PARK9	Hs00223032_m1
Parkin	PARK2	Hs00247755_m1
PINK1	PARK6	Hs00260868_m1
RIMS1	PARK10	Hs00394168_m1
LRRK2	PARK8	Hs00411197_m1
DJ-1	PARK7	Hs00697109_m1
SLC6A3	DAT	Hs00997364_m1
UBE2K	UBE2K	Hs01001790_m1
TH	TH	Hs01002184_m1
KCNJ6	GIRK2	Hs01040524_m1
SNCA	PARK1	Hs01103386_m1
GUSB	GUSB	Hs99999908_m1

Results

There are several approaches to the analysis of microarray data (summarized in Miller and Federoff, 2005). A common way is

clustering genes according to fold changes and their relevance to biological function. In the current study, we employed a three-pronged approach:

- (1) Derivation of gene lists using SAM- and ANOVA-based data analysis (see Material and methods section for details);
- (2) Analysis of candidate genes associated with cellular pathways relevant to Parkinson's disease pathology according to published literature; and
- (3) Comparison with microarray data available from previous studies.

Because the statistical inclusionary criteria for deriving differentiated gene lists are somewhat arbitrary, we used different cut-offs and methods to generate corresponding lists of genes for similar class comparisons, and then assessed the consensus of the enrichment levels among these lists at functional pathway or gene set level (see details in 'Material and methods' section). We believe that the pathway-level enrichment, which considers gene sets or pathways with multiple relevant genes rather than individual genes, would be more consistent across these gene lists. Consequently, the gene sets or functional terms would be more relevant to the underlying biology represented by the class comparison: Parkinson's disease versus normal. For the more consensus pathways or gene sets (e.g. GO terms) associated genes were retrieved from the original gene lists and an example of this analysis is shown in Supplementary Fig. 2S. We found that the enrichment levels of the functional terms were highly concordant among the different gene lists. In addition, many of these lists were relevant to Parkinson's disease pathogenesis (see below) and similar to data from other published arrays (e.g. Grunblatt *et al.*, 2004; Zhang *et al.*, 2005; Cantuti-Castelvetri *et al.*, 2007). A summary of the genes is presented in Supplementary Table 1S using three-way ANOVA (A3W, FDR10). This list was instrumental for additional cluster analyses using GenMAPP 2.1 (www.genmapp.org) (Doniger *et al.*, 2003) and for generating gene clusters that are linked to Parkinson's disease pathology (see below).

Altogether, we found 465 down- and 580 up-regulated genes in the Parkinson's disease samples (Supplementary Table 1S). When the cut-off was set at greater than 1.5-fold difference, 358 out of the 465 downregulated genes fell into this group, while only 20 of the 580 upregulated genes were represented. Interestingly, the downregulated genes showed differences as high as 11.8-fold, while upregulated genes were not increased by more than 2-fold. In addition, almost all down- or upregulated genes had a strong association with neuronal function, pointing to a high stringency of the LMD collected material. A summary of the highest downregulated genes (>3-fold) with potential reference to the function of DA neurons is shown in Supplementary Table 2S. In the following, we present a detailed listing of our results according to gene groups and pathways that have been associated with the pathogenesis of Parkinson's disease.

PARK genes

Over the past decade, it has become clear that mutations in several genes are linked to familial forms of Parkinson's disease

(Cookson, 2005; Moore *et al.*, 2005). These genes are clustered in the PARK loci and, so far, PARK1 (α -Synuclein, SNCA), PARK2 (Parkin), PARK5 (UCH-L1), PARK6 (PINK1), PARK7 (DJ-1), PARK8 (LRRK2) and PARK9 (ATP13A2) have been implicated in this form of the disease (Schiesling *et al.*, 2008). Our results demonstrate a down-regulation of PARK1, 5, 6, 7, 9 and 10 with an upregulation of the PARK10 loci-linked genes RAP1GA1 and RIMS1. Interestingly, DJ-1 was one of the highest downregulated genes (-8.55534 -fold) in our entire data set (Table 2 and Supplementary Table 2S). These results are partly congruent with previously published arrays, in which down-regulation of PARK genes has also been described (Hauser *et al.*, 2005; Moran *et al.*, 2006, 2007; Moran and Graeber, 2008).

Programmed cell death

There are two major forms of apoptosis, intrinsic and extrinsic. While the intrinsic mechanisms are linked to several stress-related dysfunctions of cellular organelles, extrinsic apoptosis is mediated by death receptors. We found a striking downregulation of PINK1 and DJ-1, ATF4 as an indicator of ER stress (Ron and Walter, 2007; Burke, 2008), several clusters of genes linked to mitochondrial impairment (see below), and downstream factors that are involved in anti- and pro-apoptotic regulation, such as the bcl-2 protein family members BCL2L1 and BCL2A1, mitogen-activated protein kinase 8 (jun-kinase) interacting protein 3 (MAPK8IP3), LRPPRC and NFRKB. Strikingly, there was a consistent upregulation of the death receptors FAS, TNFRSF10B and TNFRSF21 as well as genes involved in their signalling cascade, such as TRADD, TNFAIP8, TNIP2, CFLAR, CASP8 and NFRKB indicating that extrinsic apoptosis is activated in Parkinson's disease-affected neurons (Table 3).

Mitochondrial dysfunction and protein degradation

Inhibition of mitochondrial function and the impairment of the UPS have long been linked to Parkinson's disease pathology and are part of the intrinsic mechanisms of PCD (Bredesen *et al.*, 2006; Gomez *et al.*, 2007). Mitochondrial dysfunction is mainly characterized by the generation of reactive oxygen species (ROS), a decrease of mitochondrial complex I activity, cytochrome-c

release, ATP depletion and caspase 3 activation. We found differential expression of multiple genes related to these signaling cascades (Table 3) and consistent with other results, downregulation was more prominent confirming reduced mitochondrial activity in Parkinson's disease (Duke *et al.*, 2006). For example, there was downregulation of superoxide dismutase 1 (SOD1) and upregulation of glutathione S-transferase A1 (GSTA1), which are both implicated in protecting cells from ROS and the products of peroxidation (Raza *et al.*, 2002; Martin *et al.*, 2007), though SOD1 has recently also been shown to increase the production of toxic ROS in the intermembrane space of mitochondria (Goldsteins *et al.*, 2008). The expression of several cytochrome c oxidase subunits was also markedly decreased as well as NADH dehydrogenase subunits and the mitochondrial mRNA-binding protein LRPPRC (Mootha *et al.*, 2003).

Together with lysosomes, the UPS is part of the proteolytic machinery to degrade misfolded, damaged proteins, or proteins with an abnormal amino acid sequence. Defects in the proteolytic systems lead to accumulation and organization of cellular aggregates, such as Lewy bodies in the Parkinson's disease DA neurons (Olanow and McNaught, 2006). Our data demonstrate downregulation of gene clusters linked to ubiquitination (including the PARK genes HIP2, UCHL-1 and RAP1GA1, see above), chaperone function (e.g. heat shock and associated proteins), and subunits of the proteasome (Table 4). In this context, we also found decreased expression of ST13, a cofactor of heat-shock protein 70 (HSP70) that stabilizes its chaperone activity.

Synaptic dysfunction

There was a number of deregulated genes which are involved in synaptic function and altogether there was more down- than upregulation (Table 5). In particular, expression of synaptogyrin 3 (SYNGR3) and NSF was diminished, which has also been described in a MPTP mouse model of Parkinson's disease (Miller *et al.*, 2004). In contrast to Miller and Federoff (Miller and Federoff, 2005), we did not detect a down-regulation of the DAT-binding protein syntaxin-1A (Lee *et al.*, 2004). However, we found down-regulation of the GABA transporter member 1 (SLC6A1), GABA receptor beta subunit 1 (GABRB1) and the GABA receptor-associated proteins (GABARAPL) 1, 2 and 3 (Table 6).

Table 2 Genes associated with Parkinson's disease linkage (PARK loci)

PARK	Gene symbol	GenBank ID	Description	Fold change	P-value
PARK1	SNCA	BG260394	Synuclein, alpha (non A4 component of amyloid precursor)	-1.85899	0.00037
PARK5	UCH-L1	NM_004181	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	-1.94417	0.00409
	HIP2	NM_005339	Huntingtin interacting protein 2	-1.22173	0.00218
PARK6	PINK1	AF316873	PTEN induced putative kinase 1	-2.15839	0.00010
PARK7	DJ-1	NM_007262	Parkinson disease (autosomal recessive, early onset) 7	-8.55534	0.00048
PARK9	ATP13A2	NM_022089	ATPase type 13A2	-1.37797	0.00432
PARK10	RAP1GA1	AB007943	RAP1 GTPase activating protein	1.42168	0.00045
	RIMS1	AF263310	Regulating synaptic membrane exocytosis 1	1.22118	0.00142
	RIMS3	NM_014747	Regulating synaptic membrane exocytosis 3	-2.88055	0.00132

Table 3 Genes associated with PCD and mitochondrial function

Gene symbol	GenBank ID	Description	Fold change	P-value
Extrinsic pathway				
CASP8	NM_001228	caspase 8, apoptosis-related cysteine peptidase	1.21115	0.0033
CFLAR	AF015451	CASP8 and FADD-like apoptosis regulator	1.15656	0.0038
FAS	Z70519	Fas (TNF receptor superfamily, member 6)	1.23956	0.0013
LMNB1	NM_005573	lamin B1	1.18341	0.001
NFRKB	NM_006165	nuclear factor related to kappaB binding protein	1.19163	0.0037
TNFAIP8	BC005352	tumor necrosis factor, alpha-induced protein 8	1.18175	0.0032
TNFRSF10B	BC001281	tumor necrosis factor receptor superfamily, member 10b	1.36905	0.0005
TNFRSF21	BE568134	tumor necrosis factor receptor superfamily, member 21	1.19511	0.0037
TNIP2	AA522816	TNFAIP3 interacting protein 2	1.2128	0.0038
TRADD	L41690	TNFRSF1A-associated via death domain	1.31697	0.0034
ER – associated pathway				
ATF4	NM_001675	activating transcription factor 4	−2.00755	0.0024
Intrinsic pathway and mitochondrial dysfunction				
ABL1	NM_005157	v-abl Abelson murine leukemia viral oncogene homolog 1	−1.49544	0.0034
API5	NM_006595	apoptosis inhibitor 5	−1.18448	0.0036
BCL2L1	AL117381	BCL2-like 1	−1.45305	0.004
BCLAF1	NM_014739	BCL2-associated transcription factor 1	−1.39014	0.0012
ERCC2	A1918117	excision repair cross-compl. rodent repair deficiency, compl.	1.22115	0.0021
FOXO3	N25732	forkhead box O3	−1.87088	0.0004
GSTA1	AL096729	Glutathione S-transferase A1	1.2062	0.0041
LRPPRC	M92439	leucine-rich PPR-motif containing	−1.95934	0.0015
MAPK6	NM_002748	mitogen-activated protein kinase 6	−1.75607	0.0003
MAPK8IP3	AB028989	mitogen-activated protein kinase 8 interacting protein 3	−2.29438	0.0036
PDCD2	AA764988	programmed cell death 2	1.26341	0.0042
PDCD6	NM_013232	programmed cell death 6	−1.22536	0.0036
PPM1F	D86995	protein phosphatase 1F (PP2C domain containing)	−1.43727	0.0024
PPP2CA	BC000400	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha	−1.84478	0.0003
PPP2CB	NM_004156	protein phosphatase 2 (formerly 2A), catalytic subunit, beta	−2.00412	0.0015
PPP5C	NM_006247	protein phosphatase 5, catalytic subunit	1.31025	0.0031
PRKCA	A1471375	protein kinase C, alpha	−1.54495	0.0032
SOD1	NM_000454	superoxide dismutase 1, soluble (ALS 1 adult)	−3.39997	0.0017
SPHK2	AA485440	sphingosine kinase 2	−1.76387	0.0039
TEGT	NM_003217	testis enhanced gene transcript (BAX inhibitor 1)	−2.12068	0.0031
ATP5A1	A1587323	ATP synthase, H+ transport., mitochon. F1 complex, alpha 1	−2.29564	0.001
ATP5G3	NM_001689	ATP synthase, H+ transport., mitochon. F0 complex, subunit C3	−2.22158	0.0021
ATP5H	AF061735	ATP synthase, H+ transport., mitochon. F0 complex, subunit d	−1.65063	0.0008
ATP5J	NM_001685	ATP synthase, H+ transport., mitochon. F0 complex, subunit F6	−2.57595	0.0003
ATP5L	NM_006476	ATP synthase, H+ transporting, mitochondrial F0 complex, G	−1.33854	0.0025
CA5A	NM_001739	carbonic anhydrase VA, mitochondrial	1.1286	0.0022
COX5B	NM_001862	cytochrome c oxidase subunit Vb	−1.85634	0.0013
COX6C	NM_004374	cytochrome c oxidase subunit Vic	−2.04083	0.0044
COX7A2L	NM_004718	cytochrome c oxidase subunit VIIa polypeptide 2 like	−2.02931	0.0002
COX7C	NM_001867	cytochrome c oxidase subunit VIIc	−3.00246	0.0007
COX8A	NM_004074	cytochrome c oxidase subunit 8A (ubiquitous)	−1.7393	0.0019
FH	NM_000143	fumarate hydratase	−1.37515	0.0021
GK3P	AA292874	glycerol kinase 3 pseudogene	1.18797	0.0002
GK	AJ252550	glycerol kinase	1.29147	0.0046
GLS	NM_014905	glutaminase	−6.01418	0.0004
GPD2	U79250	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	1.23037	0.0013
HSPE1	NM_002157	heat shock 10kDa protein 1 (chaperonin 10)	−1.424	0.00003
IMMT	NM_006839	inner membrane protein, mitochondrial (mitofilin)	−2.16309	0.0009
LARS	NM_020117	leucyl-tRNA synthetase	1.51549	0.0033
LARS2	D21851	leucyl-tRNA synthetase 2, mitochondrial	−1.45482	0.0012
LARS2	D21851	leucyl-tRNA synthetase 2, mitochondrial	−1.45482	0.0012
MTCH1	AF189289	mitochondrial carrier homolog 1 (C. elegans)	−2.83831	0.0035
MRPL15	NM_014175	mitochondrial ribosomal protein L15	−1.34774	0.0014

(continued)

Table 3 Continued

Gene symbol	GenBank ID	Description	Fold change	P-value
MRPL3	BC003375	mitochondrial ribosomal protein L3	-1.98992	0.0009
MRPL34	AB049652	mitochondrial ribosomal protein L34	-1.32983	0.0028
MRPL40	NM_003776	mitochondrial ribosomal protein L40	-1.27334	0.0012
MRPL9	AB049636	mitochondrial ribosomal protein L9	1.26286	0.0002
NDUFA1	NM_004541	NADH dehydrog. (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa	-2.08152	0.0042
NDUFA4	NM_002489	NADH dehydrog. (ubiquinone) 1 alpha subcomplex, 4, 9kDa	-1.63321	0.0004
NDUFA6	NM_002490	NADH dehydrog. (ubiquinone) 1 alpha subcomplex, 6, 14kDa	-2.4391	0.0013
NDUFAB1	NM_005003	NADH dehydrogenase (ubiquinone) 1 α/β subcomplex, 1, 8kDa	-2.29908	0.0009
NDUFB2	NM_004546	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2, 8kDa	-3.70075	0.002
NDUFB3	NM_002491	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa	-2.43627	0.0023
NDUFB4	NM_004547	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	-2.02087	0.0011
NDUFB8	NM_005004	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa	-7.45941	0.0001
NDUFB11	NM_019056	NADH dehydrogenase (ubiquinone) 1 β subcomplex11, 17.3kDa	-1.45351	0.0037
NDUFC1	NM_002494	NADH dehydrog. (ubiquinone) 1 subcomplex unknown, 1, 6kDa	-1.82726	0.00004
NDUFS5	NM_004552	NADH dehydrogenase (ubiquinone) Fe-S prot. 5, 15kDa	-2.852	0.0029
NDUFS5	NM_004552	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa	-2.852	0.0029
OAT	NM_000274	ornithine aminotransferase (gyrate atrophy)	-1.76667	0.0005
OAZ3	AW611641	ornithine decarboxylase antizyme 3	1.15114	0.0018
ODC1	NM_002539	ornithine decarboxylase 1	-1.55264	0.0001
PCCB	NM_000532	propionyl Coenzyme A carboxylase, beta polypeptide	-1.16783	0.0006
SDHC	BG110532	succinate dehydrog. complex, subunit C, int. mem. prot., 15kDa	-1.98456	0.0012
SFXN3	NM_030971	sideroflexin 3	-1.75734	0.0013
SUMO3	NM_006936	SMT3 suppressor of mif two 3 homolog 3 (S. cerevisiae)	-2.68799	0.0002
TIMM17A	AK023063	translocase of inner mitochondrial mem. 17 homolog A (yeast)	-2.59562	0.0021
TOMM20	BG165094	translocase of outer mitochondrial membrane 20 homolog (yeast)	-1.09291	0.0007
UCRC	NM_013387	ubiquinol-cytochrome c reductase complex (7.2 kD)	-1.94979	0.0004
UQCRC2	NM_003366	ubiquinol-cytochrome c reductase core protein II	1.18331	0.001
UQCRH	NM_006004	ubiquinol-cytochrome c reductase hinge protein	-2.87814	0.0001

Table 4 Genes associated with protein degradation

Gene symbol	GenBank ID	Description	Fold change	P-value
SNCA	BG260394	synuclein, alpha	-1.859	0.0003
ATP13A2	NM_022089	ATPase type 13A2	-1.378	0.0043
HSF1	NM_005526	heat shock transcription factor 1	-1.4953	0.0005
HSF2BP	NM_007031	heat shock transcription factor 2 binding protein	1.23959	0.0003
HSP90AA1	R01140	heat shock protein 90kDa alpha (cytosolic), class A member 1	-5.8721	0.0026
HSPA8	AA704004	heat shock 70kDa protein 8	-2.3571	0.0033
HSPE1	NM_002157	heat shock 10kDa protein 1 (chaperonin 10)	-1.424	0.00003
HSPH1	NM_006644	heat shock 105kDa/110kDa protein 1	-1.5953	0.0038
DNAJC4	NM_005528	DnaJ (Hsp40) homolog, subfamily C, member 4	1.15186	0.0046
DNAJC7	NM_003315	DnaJ (Hsp40) homolog, subfamily C, member 7	-1.80219	0.0002
UBB	NM_018955	ubiquitin B	-5.9404	0.002
UBE1C	AL117566	ubiquitin-activating enzyme E1C (UBA3 homolog, yeast)	-1.86447	0.0012
UBE2E1	AL518159	ubiquitin-conjugating enzyme E2E 1 (UBC4/5 homolog, yeast)	-2.05043	0.0012
UBE3B	AL096740	ubiquitin protein ligase E3B	1.16931	0.0043
USP10	BC000263	ubiquitin specific peptidase 10	1.29572	0.0028
USP34	AB018272	ubiquitin specific peptidase 34	-1.9483	0.0032
USP34	AW502434	ubiquitin specific peptidase 34	1.20076	0.000008
USP47	BE966019	ubiquitin specific peptidase 47	-2.4653	0.0024
UCHL1	NM_004181	ubiquitin carboxyl-terminal esterase L1	-1.9442	0.004
UBA52	AF348700	ubiquitin A-52 residue ribosomal protein fusion product 1	-2.1443	0.0045
SCRN1	NM_014766	secernin 1	-2.09163	0.0017
CPE	NM_001873	carboxypeptidase E	-2.75212	0.0016

(continued)

Table 4 Continued

Gene symbol	GenBank ID	Description	Fold change	P-value
DNPEP	NM_012100	aspartyl aminopeptidase	−1.0922	0.0006
ADAMDEC1	NM_014479	ADAM-like, decysin 1	1.17513	0.00009
PSEN2	U34349	presenilin 2 (Alzheimer disease 4)	−2.53079	0.0009
HIP2	NM_005339	huntingtin interacting protein 2 (ubiquitin-conjugating enzyme)	−1.2217	0.0021
PSMB4	NM_002796	proteasome (prosome, macropain) subunit, beta type, 4	−2.3662	0.0046
PSMB5	BC004146	proteasome (prosome, macropain) subunit, beta type, 5	−1.5774	0.0009
PSMC3	AL545523	proteasome (prosome, macropain) 26S subunit, ATPase, 3	1.1355	0.0013
PSMD4	NM_002810	proteasome (prosome, macropain) 26S subunit, non-ATPase, 4	−2.2385	0.000009
PSMC3IP	NM_013290	PSMC3 interacting protein	1.17677	0.0033
SUMO3	NM_006936	SMT3 suppressor of mif two 3 homolog 3 (<i>S. cerevisiae</i>)	−2.68799	0.0002
AP3B2	NM_004644	adaptor-related protein complex 3, beta 2 subunit	1.24563	0.0031
AP4E1	AB030653	adaptor-related protein complex 4, epsilon 1 subunit	1.12576	0.0012
AP4S1	BC001259	adaptor-related protein complex 4, sigma 1 subunit	1.31131	0.0034
HSPC152	NM_016404	hypothetical protein HSPC152	−1.9457	0.001
GULP1	AK023668	GULP, engulfment adaptor PTB domain containing 1	1.18592	0.0014
ZFYVE9	NM_007323	zinc finger, FYVE domain containing 9	1.45751	0.0003
ATP6V0A1	AL096733	ATPase, H ⁺ transporting, lysosomal V0 subunit a1	−1.90086	0.001
ATP6V0A2	AW444520	ATPase, H ⁺ transporting, lysosomal V0 subunit a2	1.34269	0.0038
ATP6V1E1	BC004443	ATPase, H ⁺ transporting, lysosomal 31kDa, V1 subunit E1	−3.69945	0.0004

DA phenotype, survival and cytoskeleton

Interestingly, from the 1046 genes in our data set none of the 'classical' DA neuron-associated genes were significantly deregulated (e.g. TH, AADC, DAT, EN-1, NURR1), although there was a trend for reduced expression of TH and DAT by qRT-PCR (see below). We noticed an upregulation of a cluster of genes linked to cell survival (Table 6) indicating the activation of compensatory mechanisms in response to cell stress. These genes comprise mitogen-activated protein kinases (MAP3K3, MAP6, MAPK8IP3), growth factors (FGF21 and 23, GDF3, TDGF1/3), growth factor receptors and associated proteins (FGFR2, TGFBR3, NGFR, GFRA2, TNFRSF16, GDF3, DRD1, VDR), and other ion or neurotransmitter receptors (discussed separately below). In addition, there was downregulation of genes related to cytoskeletal maintenance (Table 5), e.g. dyneins, which are involved in the trafficking of cellular components, transport of organelles, cell–cell contact and cytoskeletal stability via interaction with β -catenins and microtubules. Strikingly, we found deregulation of microtubulin-associated genes like MAPT, MAPRE1, TCP1 [which take part in unfolding translated proteins in the cytosol, such as actin and tubulin (Stirling *et al.*, 2007)] and multiple subunits of tubulin (Table 5), but not microtubule affinity regulating kinase (MARK1) and microtubule-associated protein (MAP2) as described elsewhere (Miller *et al.*, 2006; Moran *et al.*, 2007).

Ion channels and neurotransmitter receptors

Over the past years, there has been emerging evidence that survival of DA neurons depends on their unique properties of

electrical activity involving Na⁺, K⁺ and Ca²⁺ channels and the association of mitochondrial dysfunction and ROS production with K⁺ and Ca²⁺ channel activation has been suspected as a major contributor to Parkinson's disease pathogenesis (Michel *et al.*, 2007; Surmeier, 2007). Many molecules related to these mechanisms are dysregulated in our data set (Table 6). For example, there was striking downregulation of the Na⁺/K⁺-ATPase carrier protein (ATP1B1), which is involved in actively pumping Na⁺ out of and K⁺ into the cell plasma to maintain their electrochemical gradients. Another downregulated gene was the G protein-gated inwardly rectifying K⁺ channel 2 (GIRK2 or KCNJ6), which is predominantly expressed in the SNc DA neurons and has been implicated in Parkinson's disease (Kobayashi and Ikeda, 2006). In addition, the calcium channel subunit β 3 (CACNB3), ATPase type 13A2 (PARK9, Table 2) and several subunits of Ca²⁺ transporting ATPases (ATP2A3, ATP2B2, ATP2C1) were downregulated further substantiating a deficit in organelle function and Ca²⁺ sequestering. Finally, our data demonstrate an upregulation of the glutamate receptors GRIN2B and GRM7 and the nicotinic cholinergic receptors α 4 and β 2 (CHRNA4, CHRN2) (Table 6), which is consistent with the notion that NMDA and nicotinic acetylcholine (ACh) receptors contribute to DA neuronal survival (reviewed in Michel *et al.*, 2007).

Validation of microarray data by TaqMan[®]-based real-time PCR

To validate the results from the microarray assays, we additionally performed TaqMan[®]-based real-time PCR on laser-microdissected cells from two new control and one new Parkinson's disease brain as well as control brain C3 and Parkinson's disease brains PD3 and PD4, which were used for the microarray analysis (Table 1). We selected the DA neuronal-specific genes tyrosine hydroxylase (TH),

Table 5 Genes associated with synaptic function

Gene symbol	GenBank ID	Description	Fold change	P-value
Transport of peptide-containing vesicles to neuron terminal				
KIF5B	BF223224	kinesin family member 5B	−1.59355	0.0041
KIF5C	NM_004522	kinesin family member 5C	−4.92852	0.0003
KIF4A	NM_012310	kinesin family member 4A	1.15103	0.0013
Vesicle reserve pool maintenance and vesicle mobilization				
SYN1	H19843	synapsin I	−1.66303	0.0004
ABLIM3	NM_014945	actin binding LIM protein family, member 3	−1.35052	0.00007
Docking				
GTPBP4	NM_012341	GTP binding protein 4	−1.53253	0.003
Priming				
NSF	NM_006178	N-ethylmaleimide-sensitive factor	−3.23079	0.0003
SV2A	NM_014849	synaptic vesicle glycoprotein 2A	−2.44187	0.0018
SV2B	NM_014848	synaptic vesicle glycoprotein 2B	−2.94679	0.0002
SNPH	NM_014723	syntaphilin	−1.45497	0.0008
RIMS1	AF263310	regulating synaptic membrane exocytosis 1	1.22118	0.0014
RIMS3	NM_014747	regulating synaptic membrane exocytosis 3	−2.88055	0.0013
CADPS	NM_003716	Ca ²⁺ -dependent secretion activator	−1.47948	0.0016
Fusion				
SYT1	AV731490	synaptotagmin I	−4.13271	0.0026
SYT12	AK024381	synaptotagmin XII	1.31759	0.0039
Coating				
CLTA	NM_001833	clathrin, light chain (Lca)	−1.75741	0.0016
CLTC	NM_004859	clathrin, heavy chain (Hc)	−4.10273	0.0001
SNPH	NM_014723	syntaphilin	−1.45497	0.0008
Budding				
DNM1	AF035321	dynamitin 1	−5.37261	0.0031
DNM2	NM_004945	dynamitin 2	−1.20722	0.0039
SYNJ2	AK026758	synaptojanin 2	1.31697	0.0022
Synaptic vesicle surface proteins				
SCAMP1	NM_004866	secretory carrier membrane protein 1	1.29675	0.0023
STX8	NM_004853	syntaxin 8	−1.34458	0.0012
SYT1	AV731490	synaptotagmin I	−4.13271	0.0026
SYP	U93305	synaptophysin	−1.6632	0.0015
VAMP4	NM_003762	vesicle-associated membrane protein 4	1.19224	0.002
SYN1	H19843	synapsin I	−1.66303	0.0004
VAMP8	NM_003761	vesicle-associated membrane protein 8	1.1353	0.0017
Proteins involved in synaptic plasticity				
SYNGR3	NM_004209	synaptogyrin 3	−4.14138	0.0009
SNCA	BG260394	synuclein, alpha	−1.85899	0.0003
Cytoskeleton				
TUBA1A	AF141347	tubulin, alpha 1a	−6.37157	0.0017
TUBB	BC005838	tubulin, beta	−1.72028	0.002
TUBB2A	NM_001069	tubulin, beta 2A	−11.845	0.0009
TUBB2B	AL533838	tubulin, beta 2B	−3.78621	0.0018
TUBB2C	AA515698	tubulin, beta 2C	−3.04285	0.0011
TUBB2C	BC004188	tubulin, beta 2C	−2.38572	0.001
TUBB3	NM_006086	tubulin, beta 3	−3.84889	0.00003
TUBD1	BC000258	tubulin, delta 1	1.32677	0.0008
DYNC111	NM_004411	dynein, cytoplasmic 1, intermediate chain 1	−3.30122	0.0033
DYNLL1	NM_003746	dynein, light chain, LC8-type 1	−2.84963	0.00006
DYNLRB1	NM_014183	dynein, light chain, roadblock-type 1	−1.86594	0.0045

Table 6 Growth factors, receptors and ion-channels

Gene symbol	GenBank ID	Description	Fold change	P-value
Growth factor—related transcripts				
CTGF	M92934	connective tissue growth factor	1.209	0.0029
TGFBR3	NM_003243	transforming growth factor, beta receptor III	1.252	0.0005
NFATC1	U08015	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	1.386	0.0029
NFATC2IP	AA152202	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	1.186	0.0023
NFKBIL2	NM_013432	nuclear factor of kappa light polypeptide gene enhancer in B-cells	1.149	0.0014
NFRKB	NM_006165	nuclear factor related to kappaB binding protein	1.1916	0.0037
NFRKB	A1887378	nuclear factor related to kappaB binding protein	1.3201	0.0016
NGFR	NM_002507	nerve growth factor receptor (TNFR superfamily, member 16)	1.21	0.0006
NGFRAP1	NM_014380	nerve growth factor receptor (TNFRSF16) associated protein 1	−4.31	0.0004
TDGF1/3	NM_003212	teratocarcinoma-derived growth factor 1/3	1.704	0.0018
GDF3	NM_020634	growth differentiation factor 3	1.263	0.003
FGF21	NM_019113	fibroblast growth factor 21	1.084	0.0037
FGF23	NM_020638	fibroblast growth factor 23	1.334	0.00003
FGFR2	M87771	fibroblast growth factor receptor 2	1.146	0.0025
GFRA2	U97145	GDNF family receptor alpha 2	1.273	0.0014
PIK3C2G	AJ000008	phosphoinositide-3-kinase, class 2, gamma polypeptide	1.28471	0.0012
PIK3R1	A1680192	phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	−1.75559	0.0037
PIK3R2	NM_005027	phosphoinositide-3-kinase, regulatory subunit 2 (p85 beta)	1.39538	0.0004
Neurotransmitter—related transcripts				
GABRB1	NM_000812	gamma-aminobutyric acid (GABA) A receptor, beta 1	−2.95	0.0037
GABARAPL1/3	AF180519	GABA(A) receptor-associated protein like 1	−4.16	0.001
GABARAPL2	AB030710	GABA(A) receptor-associated protein-like 2	−1.53	0.0012
GRIN2B	U90278	glutamate receptor, ionotropic, N-methyl D-aspartate 2B	1.267	0.0005
GRM7	X94552	glutamate receptor, metabotropic 7	1.23274	0.0014
DRD1	X58987	dopamine receptor D1	1.24	0.0045
HTR1F	NM_000866	5-hydroxytryptamine (serotonin) receptor 1F	1.169	0.0044
CHRNA4	L35901	cholinergic receptor, nicotinic, alpha 4	1.29101	0.0028
CHRN2	NM_000748	cholinergic receptor, nicotinic, beta 2 (neuronal)	1.31787	0.0018
SSTR4	NM_001052	somatostatin receptor 4	1.17716	0.0019
Ion channel—related transcripts				
KCNA10	NM_005549	potassium voltage-gated channel, shaker-related subfamily 10	1.20794	0.001
KCNJ6	U24660	potassium inwardly-rectifying channel, subfamily J, member 6	−1.50387	0.0034
KCNK1	U90065	potassium channel, subfamily K, member 1	1.18089	0.0029
KCMF1	NM_020122	potassium channel modulatory factor 1	−2.09577	0.0021
SCN3B	AB032984	sodium channel, voltage-gated, type III, beta	−1.45019	0.0028
SCN7A	NM_002976	sodium channel, voltage-gated, type VII, alpha	1.17227	0.0004
CACNB3	U07139	calcium channel, voltage-dependent, beta 3 subunit	−2.69693	0.0036
CLCNKA/KB	NM_004070	chloride channel Ka/chloride channel Kb	1.41945	0.004
ATP13A2	NM_022089	ATPase type 13A2	−1.37797	0.0043
ATP1B1	NM_001677	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	−4.96066	0.0004
ATP2A3	Y15724	ATPase, Ca ⁺⁺ transporting, ubiquitous	−1.59098	0.0009
ATP2B2	R52647	ATPase, Ca ⁺⁺ transporting, plasma membrane 2	−1.58811	0.0018
ATP2C1	AF189723	ATPase, Ca ⁺⁺ transporting, type 2C, member 1	−1.31686	0.0005
SLC6A1	A1003579	solute carrier family 6 (GABA), member 1	−1.67856	0.0008
SLC6A2	AB022847	solute carrier family 6 (noradrenalin), member 2	1.23318	0.0033
SLC11A2	AF046997	solute carrier family 11 (prot-coupled divalent metal ion transporters)	1.18662	0.0031
SLC16A3	AL513917	solute carrier family 16, 3 (monocarboxylic acid transporter 4)	1.15939	0.0007
SLC22A17	NM_020372	solute carrier family 22 (organic cation transporter), member 17	−1.87688	0.0021
SLC24A2	NM_020344	solute carrier family 24 (Na ⁺ /K ⁺ /Ca ⁺⁺ exchanger), member 2	1.18826	0.0025
SLC24A3	NM_020689	solute carrier family 24 (Na ⁺ /K ⁺ /Ca ⁺⁺ exchanger), member 3	−1.30668	0.0017
SLC24A6	NM_024959	solute carrier family 24 (Na ⁺ /K ⁺ /Ca ⁺⁺ exchanger), member 6	1.36345	0.0014
SLC34A1	NM_003052	solute carrier family 34 (sodium phosphate), member 1	1.23666	0.0043
SLC35A1	NM_006416	solute carrier family 35 (CMP-sialic acid transporter), member A1	−1.54345	0.0001
SLC39A6	A1635449	solute carrier family 39 (zinc transporter), member 6	−2.55873	0.0019
SLC43A3	A1630178	solute carrier family 43, member 3	1.29524	0.0006

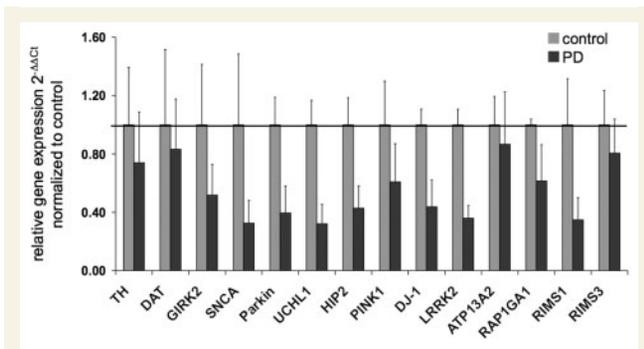


Figure 1 Validation of gene expression using TaqMan[®] real-time PCR on three control and three Parkinson's disease samples (Table 1). The following genes were selected: tyrosine hydroxylase (TH), dopamine transporter (DAT), Girk2 (KCNJ6), SNCA (PARK1), Parkin (PARK2), UCHL1 and HIP2 (PARK5), PINK1 (PARK6), DJ-1 (PARK7), LRRK2 (PARK8), ATP12A2 (PARK9), RAP1GA1, RIMS1 and 3 (PARK10). Data analysis was based on the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) and results were plotted as fold differences of relative gene expression normalized to controls.

dopamine transporter (DAT or SNC6A3) and Girk2 (KCNJ6) (Table 6) and all PARK genes including LRRK2, which was not present on the HG-U133A Affymetrix chip. Using the $2^{-\Delta\Delta C_t}$ method to determine fold differences of relative gene expression in Parkinson's disease versus control samples (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008), the real-time PCR experiments largely confirmed the results from the microarrays (Fig. 1). However, we also observed high variability between samples, which prompted us to additionally analyse our results by comparing relative gene expression of individual genes using the $2^{-\Delta C_t}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) for the real-time PCR assays and Z-scores for the microarrays after removal of batch effects (Supplementary Fig. S3). Although these data showed considerable variability of gene expression levels within each sample (Supplementary Fig. S3A) and across the sample population (Supplementary Fig. S3B), there was an overall consistency between both methodologies demonstrating a broad downregulation of PARK genes and, to some extent, also of TH and DAT in the PCR assays. The latter, however, did not reach significance in the microarrays using three-way ANOVA at FDR10%.

Discussion

Studying Parkinson's disease pathogenesis using microarray technology

Multiple microarray studies have compared the gene expression profiles of cells within the midbrain of normal controls with those from Parkinson's disease brains (Grunblatt *et al.*, 2004; Hauser *et al.*, 2005; Zhang *et al.*, 2005; Duke *et al.*, 2006; Miller *et al.*, 2006; Moran *et al.*, 2006, 2007; Moran and Graeber, 2008).

These studies were based on sections encompassing substantia nigra as well as other adjacent regions such as striatum and thalamus, and therefore, contained a large amount of cells other than DA neurons. Consequently, microarray analyses on dissected tissue revealed a global set of genes that are dysregulated in Parkinson's disease, which is in agreement with an increasing conceptual view that not only the DA neurons, but also other cells within the substantia nigra and adjacent brain regions are involved in Parkinson's disease pathology (summarized in Duke *et al.*, 2006). Altogether, these studies confirmed several cellular functions that are affected in Parkinson's disease, such as the UPS and the mitochondrial system, synapse function, DA phenotype, and cytoskeletal maintenance pointing to defects in cell communication, survival and axonal transport (Duke *et al.*, 2006; Miller *et al.*, 2006). However, they do not provide gene expression of single DA neurons. So far, three groups reported expression profiling on directly targeted DA neurons by laser microscopy (Lu *et al.*, 2004, 2006; Cantuti-Castelvetri *et al.*, 2007; Grundemann *et al.*, 2008). Two of these groups used laser capture microscopy (LCM) with an Arcturus PixCell II instrument after quick immunostaining or ethanol fixation and methylene blue staining of the dissected midbrain tissue. This differs from our and Grundemann *et al.*'s approach, in which LMD was performed on unprocessed freshly cut sections and the DA neurons visualized by their neuromelanin content. In addition, the LMD-isolated neurons fell by gravity into collection tubes, in contrast to fixation of the cells on the slide matrix by LCM. We attempted to compare our results with the microarray data published by (Cantuti-Castelvetri *et al.*, 2007), but unfortunately in this study a different Affymetrix platform with a different probe set (U133_X3P) was used (<http://www.ebi.ac.uk/microarray-as/ae/>). Based on our analysis criteria (three-way ANOVA, FDR10), we were not able to retrieve differential gene expression profiles as seen in our study.

It should be noted that an important parameter in the interpretation of the LMD-based microarray data refers to the integrity and status of the isolated cells. Especially downregulation of gene expression could be a result of neuronal death that is not necessarily related to a dysfunction of pathways associated with Parkinson's disease pathogenesis. Therefore, it should be emphasized that gene expression in this study should be viewed in the context of biological function and—when deregulated—in relation to a possible role in pathogenic processes that are linked to Parkinson's disease.

Deregulated gene expression as indicator for dysfunctional cellular pathways in Parkinson's disease

PARK genes

PARK proteins are associated with familial forms of Parkinson's disease and their functions have been linked to all major pathways related to Parkinson's disease pathogenesis including mitochondrial and synaptic dysfunction, protein degradation, PCD and cell survival (Moran *et al.*, 2007; Olanow, 2007; Thomas and Beal, 2007; Burke, 2008; Schiesling *et al.*, 2008). Although there is evidence that both forms of Parkinson's disease share common pathogenic

mechanisms, it is still unclear if, and to what extent, the familial-linked PARK proteins are involved in the sporadic illness. Our data show a striking downregulation of most of the PARK genes. Since PARK1, RIMS1 and RIMS3 are involved in vesicular function, PARK2, PARK5 and RAP1GA1 with the UPS, PARK6 in mitochondrial function, PARK7 in intrinsic pathways of PCD, and PARK8 in cytoskeletal process regulation, it appears that a deregulation of these molecules might also contribute to the pathogenesis of sporadic Parkinson's disease. Thus, our data could support the view that the PARK genes might present a significant group of key factors in common pathogenetic mechanisms of both forms of Parkinson's disease (Moran *et al.*, 2007; Thomas and Beal, 2007; Burke, 2008; Schiesling *et al.*, 2008).

Cellular pathways involved in Parkinson's disease pathogenesis

Multiple cellular pathways have been associated with Parkinson's disease pathogenesis and one of the key mechanisms relates to processes involved in PCD. These comprise a large subset of molecules that also include some of the PARK genes, such as PARKIN, PINK1 and DJ-1 (Tatton *et al.*, 2003; Burke, 2007, 2008; Moran *et al.*, 2007; Olanow, 2007; Singh and Dikshit, 2007; Schiesling *et al.*, 2008). Many of the functional aspects of these molecules stem from experimental models of Parkinson's disease and have been extensively summarized elsewhere (e.g. Olanow, 2007; Singh and Dikshit, 2007; Burke, 2008). However, there is only very little information available from Parkinson's disease patient's material other than rather controversial and mixed results from morphologic assessments (Tatton *et al.*, 2003; Burke, 2007, 2008). Our data show a set of deregulated genes that are directly or indirectly involved in PCD confirming the current concept of apoptotic cell death of the DA neuron. Particularly interesting is the observed upregulation of genes involved in extrinsic PCD, because there have been several observations on postmortem brain tissue suggesting a role of TNF- α and FAS signalling in the neurodegeneration of Parkinson's disease (Boka *et al.*, 1994; Mogi *et al.*, 1996; Ferrer *et al.*, 2000; Hartmann *et al.*, 2001, 2002; Burke, 2007). In addition, our data show a dysfunction of both the mitochondria and the UPS, which are major contributors to PCD and Parkinson's disease pathogenesis (Duke *et al.*, 2006). This included multiple cytochrome c oxidase and NADH dehydrogenase subunits that have been recently associated with impaired mitochondrial function in pesticide-induced Parkinson's disease (Gomez *et al.*, 2007). Interestingly, there was a decrease of LRPPRC expression, a gene linked to the mitochondrial neurodegenerative disorder French-Canadian-type Leigh syndrome, which is caused by defects in oxidative phosphorylation (Mootha *et al.*, 2003) and ST13, which is part of a number of marker genes (including HIP2) that have been proposed as possible biomarkers in Parkinson's disease (Scherzer *et al.*, 2007). It should be noted that SNCA, a component of Lewy bodies, whose pathologic accumulation is caused by oxidative stress, mitochondrial dysfunction and impairment of cellular proteolytic mechanisms (Lundvig *et al.*, 2005) was also deregulated.

There were several deregulated genes pointing to impairment of synaptic function and plasticity and some of these genes were also observed in other studies, such as SYNGR3, NSF, SV2B, SYN1,

SYT1 and dynamin (Miller *et al.*, 2006). The deregulated genes in our study belong to important mechanisms involved in maintaining synaptic function and integrity, such as a number of proteins from the SNARE complex (*priming of the synaptic vesicle and synaptic vesicle surface proteins*) that play a role in vesicle binding and fusion to the plasma membrane (Brunger, 2005). Other downregulated genes encode the GTPase family-associated molecules dynamin 1 and 2, which are involved in severing nascent vesicles from the membrane, receptor-mediated endocytosis, trafficking in and out of the Golgi apparatus, maintenance of mitochondrial morphology and mitochondrial-associated pathways of apoptosis (Scorrano, 2007; Ungewickell and Hinrichsen, 2007). In addition, there was striking down-regulation of genes related to cytoskeletal maintenance including MAP kinases, tubulins and dyneins, while several growth factor receptor and their signalling-associated genes were upregulated. We also found downregulation of GABA receptor and signalling-related genes supporting the previous suggestion that GABAergic synapses are reduced in the substantia nigra of Parkinson's disease resulting in a reduction of DA neuron inhibition and an increase in neurotransmission and function of the remaining functional DA neurons (see below) (Miller and Federoff, 2005). Altogether our results are consistent with other observations pointing to a functional disconnect of the striatonigral trophic signalling pathways (Miller *et al.*, 2006).

Our data also support evidence from other investigators suggesting that survival of DA neurons depends on their unique properties of electrical activity involving Na⁺, K⁺ and Ca²⁺ channels. For example, Michel *et al.* proposed a mechanism in which the dysfunctional mitochondria and ROS trigger adenosine triphosphate-sensitive K⁺ (K_{ATP}) channel-mediated hyperpolarization of substantia nigra DA neurons, which renders them susceptible to degeneration (Michel *et al.*, 2007). We found a striking downregulation of the Na⁺/K⁺-ATPase carrier protein (ATP1B1) that is involved in actively pumping Na⁺ out of and K⁺ into the cell plasma to maintain their electrochemical gradient. Mutation in this gene causes rapid-onset dystonia Parkinsonism (de Carvalho Aguiar *et al.*, 2004). It should be noted that SOD (or SOD mimetics) can abolish the K⁺-mediated hyperpolarization by inhibiting ROS formation (Liss *et al.*, 2005) and expression of SOD was markedly downregulated in our data. Also, there was downregulation of GIRK2 expression, which can cause permanent depolarization and loss of spontaneous pacemaker activity and, thus, contributes to cell death (Liss *et al.*, 2005). Other receptors that have been implicated in the long-term survival of DA neurons are L-type Ca²⁺ channels, which drive their pace-making activity by sustaining low intracellular Ca²⁺ concentrations that are sequestered by the ER and mitochondria using ATP-dependent transporters (Surmeier, 2007). These energy-consuming processes require oxidative phosphorylation, a prominent feature of DA neurons. In combination with the generation of ROS and consecutive mitochondrial DNA damage this high metabolic rate might accelerate their ageing—including dysfunctional proteins that are directly or indirectly involved in these processes, e.g. some of the PARK genes including ATPase type 13A2 (Surmeier, 2007). Our data show a reduction in multiple calcium channel subunits including ATPase type 13A2 (PARK9) and several subunits of Ca²⁺ transporting ATPases adding to the overall picture of an imbalanced

Ca²⁺ homeostasis of the Parkinson's disease DA neuron. Finally, neurotransmitters have also been implicated in the survival of DA neurons (reviewed in Michel *et al.*, 2007). NMDA receptors seem to be involved in controlling their burst-firing mode and enhance the survival promoting effect of BDNF. However, there is also evidence that they contribute to degeneration through an excitotoxic process. Nicotinic ACh receptors protect DA neurons *in vitro* and *in vivo* against MPTP or 6-OHDA toxicity and their effects are attributed to a reduction of glutamate-mediated excitotoxicity, upregulation of trophic factors, or a rise in intracellular Ca²⁺ (see above). This is particularly interesting, since the ACh receptors $\alpha 7$, $\alpha 4$ and $\beta 2$ have strong depolarizing activity on DA neurons consistent with the view that modulation of their excitability might support survival (Matsubayashi *et al.*, 2004; Quik *et al.*, 2007). Taken together, the upregulation of glutamate nicotinic cholinergic receptors in our data set contributes to the interpretation that compensatory survival mechanisms are activated in response to cell stress mediated by PCD, protein degradation, mitochondrial and synaptic dysfunction.

Insights into Parkinson's disease pathogenesis through a 'molecular fingerprint' identity of the parkinsonian DA neuron

Miller and Federoff postulated a model for common pathways of Parkinson's disease pathogenesis based on microarray data collection (Miller and Federoff, 2005). This model encompasses several genes that are involved in the function or dysfunction of DA neurons in Parkinson's disease model systems and postmortem brain analyses from Parkinson's disease patients. Downregulated genes are related to the DA phenotype, synaptic function, cytoskeletal stability and axonal transport, while upregulated genes refer to metabolism, protein disposal and inflammation. Among the postulated genes, we found no significant down- or upregulation of DAT, AADC, EN1, MARK-1, MAP2, DSCR1L1, HK1, ZFP162 and UNC-5. However, and also consistent with other reports, there was a downregulation of SYNGR3 (Miller and Federoff, 2005), Synaptotagmin 1 (SYT1) (Zhang *et al.*, 2005; Moran *et al.*, 2006), *N*-ethylmaleimide-sensitive factor (NSF) (Miller and Federoff, 2005; Zhang *et al.*, 2005), UCHL-1 (Moran *et al.*, 2007), kinesin family members (KIF5B and KIF5C) (Miller *et al.*, 2006), and dynein-related genes (DYNC111, DYNLL1 and DYNLRB1) (Miller and Federoff, 2005). Although several of these genes are linked to pathways in DA pathogenesis (see above), we could not confirm the six genes in the Miller and Federoff study (Miller and Federoff, 2005), which are postulated as a highly conserved dysregulation in the three Parkinson's disease systems analysed (i.e. DAT, EN-1, HK-1, DSCR1L1, ZFP 162 and UNC-5). Given that many of their cellular functions in DA neurons are currently unknown (except of DAT and EN-1) further studies will be needed to confirm their direct or indirect involvement in Parkinson's disease pathology.

A recent publication by Moran and Graeber (2008) provided an extensive pathways analysis based on 892 dysregulated priority genes from a Parkinson's disease substantia nigra microarray

data set. The authors concluded that Parkinson's disease has biological associations with cancer, diabetes, and inflammation. In addition, this study revealed prominent changes in similar cell function and disease pathways evident from our data, such as apoptosis, cell survival, cytoskeleton, signal transduction, synaptic and mitochondrial function, protein degradation and networks that are directly linked to Parkinson's disease-associated genes. These investigators also found a strong association with inflammation and, interestingly, a cluster of upregulated genes related to functions of the immune system are also present in our data set (Supplementary Table 3S). This might add further evidence to an involvement of inflammatory processes in the disease development of Parkinson's disease (Whitton, 2007). Altogether, comparison of our results with the data from these and other investigators as discussed above suggests that there are two major classes of factors involved in Parkinson's disease pathogenesis:

- (1) A core of highly conserved primary (priority) factors that are major players of key pathways in the function of the substantia nigra DA neuronal phenotype; and
- (2) Secondary factors that are directly or indirectly affected by (or effect) the dysfunction of the primary molecules. Dysregulation of molecules from both classes contribute to Parkinson's disease.

It is important to emphasize that mRNA data reveal information about transcriptional activation of genes, but do not tell much about actual protein levels and function. In addition, array data cannot predict if deregulated gene expression is a primary or a secondary effect of cell function. For example, a gene could be down- or upregulated by factors, such as miRNAs or transcriptional activators (or inhibitors) independent of its protein function and/or as a consequence of positive and negative feedback loops. Moreover, protein function relies on the interaction of down- and upstream factors within a pathway, i.e. downstream factors are more dependent on upstream signalling than upstream factors, which might influence a cascade of downstream events that can include multiple pathways. Thus, the consequences of deregulated gene expression are on multiple levels within a complex and dynamic interplay of factors and mechanisms. Laser microscopy-based microarray studies can only reveal a 'snap-shot' of these events. Nevertheless, our study shows that many genes associated with Parkinson's disease pathogenesis are deregulated in single captured postmortem DA neurons. This could provide a 'molecular fingerprint identity' of a late stage DA neuron affected by sporadic Parkinson's disease. A key aspect is the striking downregulation of PARK genes. Since their mutation-induced malfunction in the familial forms of Parkinson's disease rapidly accelerates DA neuron degeneration, the results from our study could support the view that these genes are also involved in the pathogenesis of sporadic Parkinson's disease. Our data also point to an imbalance in the neuronal homeostasis and stress characterized by factors related to high metabolic rate, neurotransmission and ion-channel activity. This stress might be part of the DA neuronal normal homeostasis and aging, but could exacerbate when there is an unfavourable imbalance. In addition, the array data suggest a disintegration of key cellular functions, such as

mitochondria-associated energy metabolism, protein degradation, synaptic function and cytoskeletal integrity revealing a cellular state that is characterized by PCD. However, despite this cellular demise, some genes linked to survival mechanisms were upregulated indicating the activation of compensatory mechanisms. Finally, the lack of or relatively modest deregulation of genes important for the DA neuronal phenotype suggests that the DA neurotransmitter identity (including DA production) seems to be sustained even when the neurons are severely damaged. Altogether, it appears that the gene expression profile of late stage Parkinson's disease DA neurons is consistent with the view that Parkinson's disease is a complex disorder and that multiple factors and cellular pathways are involved in its pathogenesis.

Supplementary material

Supplementary material is available at *BRAIN* online.

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