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Supplemental Information

Inefficient DNA Repair Is an Aging-Related Modifier of Parkinson's Disease

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Supplemental figure legends

Figure S1. (related to Figure1) Expression of DAT-driven *Cre* recombinase is specific for DA neurons of the SNpc. (**a**) In DAT-Cre^{+}Rosa26-LacZ animals, beta-galactosidase staining co-localizes with SNpc tyrosine hydroxylase immunopositive cells. (**b**) Beta-galactosidase staining is not detectable in the tryptophan hydroxylase positive serotonergic neurons of the dorsal Raphe nucleus DRN. Scale bar; 50 μm.

Figure S2. (related to Figure 2 and 3) **(a)** The thiol/disulfide redox balance in unchanged in *Ercc1*$^{\Delta/+}$ hippocampal and Purkinje neurons, as determined by histochemical labeling of oxidized and reduced cysteines (see material and methods). Graphs represent mean ± SEM; unpaired t-test. Six 20-weeks old animals from each genotype were used for the experiment. (**b**) No changes in alpha-synuclein expression in *Ercc1*$^{\Delta/+}$ SNpc DA neurons. alpha-synuclein levels in DA neurons was measured as intensity of fluorescence in TH-positive regions, using laser scanning confocal microscopy in DA neurons. (**c**) No changes in proteasome activity in *Ercc1*$^{\Delta/+}$ brains. Graphs represent mean ± SEM; unpaired t-test. For these experiments, six 20-weeks old animals from each genotype were used.

Figure S3. (related to figure 3) Increased activation of astrocytes in *Ercc1*$^{\Delta/+}$ mice (green TH; red GFAP). (**a**) Increased GFAP immunoreactivity in the SNpc (top) and in the CPu of the striatum (bottom). The images in the striatum were obtained by 3D-reconstruction following 4PI microscopy and emphasize how astrocytic activation (red) is paralleled by decreased DA innervation (green). (**b**) 3D-reconstruction at higher magnification and subsequent zoom (inset in the right image) in *Ercc1*$^{\Delta/+}$ striata. Scale bar; (a) 150 μm (top) and 100 μm (bottom); (b) 10 μm.

Figure S4. (**a**) (related to Figure 3) qPCR performed on 12 significantly ($p<0.05$) up- or down-regulated genes randomly selected among the top altered pathways (Supplementary table 2) validate RNA-seq data.

Supplemental table legends

Table S1. (related to Figure 3) Significantly altered pathways in the ventral midbrain region of Ercc1Δ/+ mutants versus wild-type, matched controls, determined by ORA.

Table S2. (related to Figure3) Enrichment factor (EF) analysis confirms highly significant overlap between the up- and down-regulated pathways in *Ercc1*$^{\Delta/+}$ and PD datasets. Overlap was also significant between up-regulated pathways in *Ercc1*$^{\Delta/+}$ and ILBD datasets. As expected, overlap between ILBD and PD was highly significant in both up- and down-regulated pathways.

Table S3. (related to Figure 3) List of qPCR primers used to validate the RNA-seq data.

Table S4. (related to Figure 4) Demographic and clinical data of subjects involved in the study.

Supplemental experimental procedures

Human dermal fibroblast

Primary human dermal fibroblasts from healthy and idiopathic PD patients were obtained as previously described (Ambrosi et al., 2014); clinical data are summarized in Supplementary table 1. Fibroblasts from genetic PD (catalog ID ND32975, ND32976, ND32970, ND33879) were obtained from the Cell Repositories of the Coriell Institute for Medical Sciences (http://ccr.coriell.org/Sections/Search/). Fibroblast from AD patient and matched control were obtained from the Cell Repository of the Coriell Institute for Medical Sciences (catalog ID AG05810, AG07872, AG07936, AG08125, AG08269, AG08527, AG08543, AG21158). In two cases, controls were related to probands (AG07936, AG08125, and AG07872 from family 1079, AG08527 and AG08543 from family 2051. Fibroblasts were cultured according to standard procedures, at 37C, 5%CO_2 atmosphere, in DMEM medium supplemented with 20 % of fetal bovine serum.

Unscheduled DNA Synthesis assay (UDS)

Cells were irradiated with UV-C 16j/m^2 and then incubated with F10 medium supplemented with 10% dialyzed serum, 20 μM EdU and 1μM 5-Fluoro-2′-deoxyuridine (Fudr) for 3 h. Cells were then fixed in 4% paraformaldehyde (PFA) and processed for the copper(I)-catalyzed azide–alkyne cycloaddition of fluorescent azides using Click-iT® EdU Alexa Fluor® 594 Imaging Kit, Invitrogen.

Cells were imaged with an Olympus BX 51 microscope equipped with a Leica DFC 420 camera. Detection parameters were set in the control reaction and were kept constant across specimens. Images were analyzed in a semi-automated fashion with Metamorph software (MolecularDevices). The software automatically generated regions of interest (ROI) on the basis of the nuclear marker signal (DAPI).

Induction of double strand breaks in primary fibroblasts and immunofluorescence staining

Primary fibroblasts were seeded on glass coverslips at a concentration of 10^5 cells/well and exposed to a 2Gy γ-irradiation dose (Cesium-137 source; Gammacell 40 low dose rate laboratory irradiator, Nordion). Cells were then collected 1h and 24h after irradiation, fixed in 4% PFA, and processed for immunofluorescence. Briefly, fibroblasts were permeabilized with TritonX-100 0.5% for 20 minutes and subsequently incubated with BSA 3%. Cells were then incubated overnight at 4°C with mouse monoclonal anti-phospho-histone

H2AX antibody (anti-γH2AX - s139; 1:1000; 05336, Millipore)). After rinses in PBS, cells were incubated with Alexa488 conjugated donkey anti-mouse antibody (Invitrogen). Finally, the coverslips were mounted with Vectashield Mounting Medium with DAPI (H-1200; Vector) and image acquisition was performed in a Leica TCS SP5 confocal microscope. The detection parameters were set in the control samples and were kept constant across specimens. Images were analyzed in a semi-automated fashion using the Cell Profiler software (Carpenter et al., 2006).

Animals

Generation and characterization of NER mutants Ercc1$^{\Delta/+}$ mice has been previously described (Weeda et al., 1997). *Ercc1*$^{\Delta/+}$ mice were generated in an FVB:C57BL/6J (50:50) genetic background.

Conditional mutants were generated using the Cre-recombinase:loxP (Cre-loxP) system and crossing a strain carrying an Ercc1 allele flanked by loxP sites (Ercc1fl/fl), with a strain expressing the Cre recombinase under the control of the specific Dopamine Transporter (DAT) promoter (Zhuang et al., 2005). We validated the specificity of Cre recombinase activity by generating DAT-Cre+ROSA26-lacZ reporter (Soriano, 1999), which confirmed expression in DA neurons of the SNpc and not in other cell types, such as serotonergic neurons of the Raphe nucleus (Supp. fig. 1).

Animals were kept on a regular diet and housed at the Animal Resource Center (Erasmus University Medical Center), which operates in compliance with the "Animal Welfare Act" of the Dutch government, following the "Guide for the Care and Use of Laboratory Animals" as its standard.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) treatment

Mice were injected with 20 mg/kg of MPTP (M0986,Sigma-Aldrich, St. Louis, MO, USA) or saline (Vehicle) intraperitoneally (i.p.) every 2h for a total of four injections, resulting in a cumulative dose of 80 mg/kg, as previously described (Jackson-Lewis and Przedborski, 2007). Animals were sacrificed for morphological analysis 3 days after treatment.

Morphological analysis

Animals were deeply anesthetized with pentobarbital (1 g/kg body weight, injected i.p.), before being sacrificed by transcardial perfusion. Mice were perfused at RT with phosphate buffer saline (PBS), followed

by 4% PFA in 0.12M phosphate buffer, pH 7.6 After washing, brains were cryoprotected in 30% sucrose solution in 0.1M phosphate buffer (PB). Brains from each mouse were embedded in the gelatin block (12% of gelatin powder-J.T.Baker- dissolved in 10% sucrose solution) to get in the same block brain from wt and mutants. . The blocks were cut by a frozen microtome to obtain coronal 40 μm thick sections.

Immunohistochemistry

Free floating sections were incubated in a 0.3% hydrogen peroxide solution, for 10 min, protected from the light, to block internal peroxidases, and then in TBS 0.5% Triton X-100, 10% normal horse serum (NHS) for 1 h, RT. Sections were then incubated for 24 h at 4°C with sheep polyclonal anti-Tyrosine Hydroxylase (1:4000, NB300-110, Novus Biologicals), in TBS containing 0.4% Triton X-100, 2% NHS. In control sections, the primary antibody was omitted. After incubation for 2h at RT with biotinylated goat anti-sheep IgG (Sigma, B7401), 1:500 in TBS containing 0.4% Triton X100, 2% NHS, immuno-complexes were revealed by Vectastain Elite ABC kit (Vector), using 3,3'-diamino-benzidine (DAB Substrate kit for Peroxidase, Vector) as the chromogen. Finally, sections were dehydrated and mounted with Permount (Fisher Scientific). Slides were observed with an Olympus BX 51 microscope equipped with a Leica DFC 420 camera. Electronic images were captured by a Olympus Cellsens® 1.9 digital imaging.

Stereological counts

Unbiased stereological estimation of the total number of the dopaminergic cells in SNc was made using the optical fractionator method (West et al., 1991) from the STEREO INVESTIGATOR program on a Neurolucida computer-controlled microscopy system (Microbrightfield Inc., Williston, VT, USA). The edges of the SNc in the rostro-caudal axis were defined at all levels, with reference to a coronal atlas of the mouse brain (Paxinos and Franklin, 2004). TH-positive cells in the SNc of both hemispheres were counted in every three section, on comparable sections for all the subgroups of treatment throughout the entire nucleus. Counting frames (60 x 60 μm) were placed at the intersections of a grid (frame size 120 x 120 μm) that had been randomly placed over the section. Only counting frames for which at least a part of the frame fell within the contour of the SNc were used for counting. Cells were marked if they were TH-positive and were in focus within the counting area. Guard volumes (3 μm from the top and 3 μm from the bottom of the section) were excluded from both

surfaces to avoid the problem of lost caps. The reliability of this estimate was assessed by calculation of the coefficient of error according to the formulae described in West & Gundersen (West and Gundersen, 1990).

Immunofluorescence

For immunofluorescence experiments, the sections were processed as described in the immunohistochemistry section with some modifications. Sections were incubated overnight at 4°C with primary antibodies. The following antibodies were used: mouse monoclonal anti-Tyrosine Hydroxylase (1:4000, MAB318, Millipore), sheep polyclonal anti-Tyrosine Hydroxylase (1:4000; NB300-110, Novus Biologicals), anti-NeuN (1:1000, Mab377, Millipore), anti-glial fibrillary acidic protein (GFAP) (1:2000, 20334, DAKO), monoclonal anti-α-Synuclein (1:500; 4179, Cell Signaling), anti-phospho-α-Synuclein (S129 ; 1:500; ab59264, Abcam), monoclonal anti-ubiquitin (1:500; MK11-3, MBL), anti-γH2AX (s139; 1:1000; 05336, Millipore)

Sections were rinsed with PBS and then incubated for 2h at RT in TBS containing 0.4 % Triton X-100, 2% NHS, Alexa488 conjugated donkey anti-rabbit IgG 1:500 (Invitrogen), Alexa594 conjugated donkey anti-mouse IgG 1:500 (Invitrogen). Controls were performed by omitting the primary antibody. Image acquisition was performed in a Leica TCS SP5 confocal microscope. The detection parameters were set in the control samples and were kept constant across specimens. Images were analysed in a semi-automated fashion using the Metamorph software (MolecularDevices). The software automatically generated regions of interest (ROI) on the basis of the signal of the cellular marker (TH). The software measures the signal intensity of other markers within the ROIs.

Electron microscopic analysis

For ultrastructural analyses, animals were anesthetised and perfusion-fixed as above. 100-µm-thick sections were obtained by a vibratome and processed for TH immunoenzymatic localization. After incubation in blocking medium (PBS containing 10% NHS and 0.2% Triton-X 100) for 1h at RT, free floating sections were incubated for 48 h at 4°C with mouse monoclonal anti-TH (1:4000, Millipore MAB318), diluted in PBS containing 2% NHS and 0.1% Triton-X 100, under continuous agitation. Subsequent incubations with the biotinylated secondary antibody, ABC, and with DAB were carried out as described above in the immunohistochemistry section. After extensive washes in ice-cold PBS, slices were post-fixed in 1% OsO_4 in

0.12M phosphate buffer for 1 h at 4°C, rinsed in water, dehydrated in graded ethanol and propylene oxide, and flat-embedded in Epon 812 (TAAB, Reading, UK). Portions of ventral midbrain containing DA neurons were remounted on Epon blanks, and further sectioned on a Reichert Ultracut S ultramicrotome. Ultrathin sections were briefly contrasted with uranyl acetate and lead citrate, and observed in a Philips CM120 electron microscope equipped with a Philips Megaview III camera. Electronic images were captured by AnalySys 2.0 software and composed in Adobe Photoshop CS6 format.

4Pi microscopy

Sections were processed as for immunofluorescence and incubated with mouse monoclonal anti-Tyrosine hydroxylase (1:4000, Millipore MAB318) in combination with (rabbit polyclonal anti-GFAP 1:2000, DAKO 20334). Section were mounted between two quartz coverslips and observed with two HCX PL APO 100X glycerol N.A. 1.35 0.22/0.22 objective. 4Pi microscopy (Nagorni and Hell, 1998) was performed with a Leica TCS 4Pi unit equipped with argon and a Mai Tai tunable multiphoton laser. Three-dimensional reconstruction was performed with Amira 5 software (Visage Imaging GmbH).

Histochemical labelling of oxidized and reduced cysteines (redox histochemistry)

Histological labelling of thiol/disulfides was performed as previously described (Horowitz et al., 2011), with minor modifications. Briefly, animals were killed by cervical dislocation and the brain was quickly frozen. Coronal 10 μm thick sections were serially cut by cryostat and immediately fixed for 20 min with the fixation solution prepared in phosphate buffer containing 4% PFA, 0.05% TritonX-100, 1 mM N-Ethylmaleimide (NEM) and 5μM 7-Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin (CPM). A quenching step was then performed incubating the samples with 100mM NEM for 20 min. After three washes in PBS to remove excess unreacted dye, samples were a incubated 30min in 5mM tris (2-carboxyethyl)phosphine (TCEP). After three washes in PBS, the second labeling step was performed by incubating section for 20min in 1mM NEM, 5 μM Alexa 488 malemide in PBS. After three additional washing steps in PBS (three times for 5 min) sections were ready for further immunochemical staining.

Bioenergetics Assays

For bioenergetics assays, animals were killed by cervical dislocation, brains were rapidly excised on an ice-cold plate, and ventral midbrain was dissected and processed for mitochondrial extraction. The tissue was minced in 10 volume of mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5mM HEPES, 1mM EGTA and 0,05% fatty acid free bovine serum albumin), pH7.2; tissues were rinsed several times to remove blood. The tissue was then disrupted using a drill-driven Teflon dounce homogenizer and the solution was centrifuged at 800g for 10 minutes at 4°C. Following centrifugation, the supernatant was transferred in a clean tube and centrifuged again at 8000g for 10 minutes at 4°C. After removal of the supernatant, the pellet was re-suspended and the centrifugation was repeated. The final pellet was re-suspend in a minimal volume of mitochondrial isolation buffer. Total protein concentration was determined by Bradford Assay method. Respiration was monitored with a XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). 10 µg of mitochondrial protein were loaded in each well of a Seahorse plate, which was then spun 2000g for 20'at 4°C; after this step, the volume of mitochondrial isolation buffer in the wells was adjusted to 450µL and the plate incubated 8'at 37°C. Complex I driven respiration was sustained by glutamate (2mM) and malate (2mM), complex II driven respiration was sustained by succinate (10mM); in the latter case, rotenone (4µM) was added to inhibit complex I and prevent reversed electron transfer from succinate to NAD^+. State 3 was elicited by addition of 4mM ADP.

Proteasome activity

For proteasome activity, animals were killed by cervical dislocation, brains were rapidly excised on an ice-cold plate, and ventral midbrain was dissected. Tissues were processed for the 26S proteasome activity according to the methods described by Strucksberg and colleagues (Strucksberg et al., 2010) using the Proteasome-Glo™Assay System (G8621 and G8631; Promega) Briefly, tissues were homogenized in ice old PBS containing 5mM EDTA (PBSE) and the supernatant was collected after sonication and centrifugation. To detect proteasomal activity, 10 µg of soluble proteins were added to the luminescent reagent containing chymotrypsin-like or trypsin-like substrates. After 1 hour incubation at room temperature, luminescence was detected using a GloMax®-Multi Microplate Reader (Promega). Proteasomal activity was calculated by subtracting the unspecific background activity obtained by the incubation of samples with the specific proteasome inhibitor $AdaAhx_3L_3VS$ (30 µM, Calbiochem; cat. 114802) from the total peptidase activity.

Finally, the proteasomal activity was correlated to the total amount of proteasome contained in the samples by normalization using the densitometry value obtained from the 26S proteasome S4-subunit immunoblot analysis.

Messenger RNA sequencing (mRNA-Seq)

For RNA extraction, animals were sacrificed by cervical dislocation and brains were rapidly excised on an ice-cold plate. Total RNA was isolated from the ventral mesencephalic area of the mouse brain using the RNAqueous Kit (Ambion) according to manufactures directions. Briefly, ventral midbrain tissues were homogenized in RNA lysis buffer and stored at -80^C until the extraction procedure. RNA quality was assessed via the BioAnalyzer machine (Agilent 2100). Only samples with a RIN greater than 8 were used for further processing.

Total RNA was used for the messenger RNA sequencing sample preparation using the TruSeq mRNA sample preparation kit (Illumina) according to the manufacturer's protocols. Briefly, 1 µg of total RNA for each sample was used for poly(A) RNA selection using magnetic beads coated with poly-dT, followed by thermal fragmentation. The fragmented poly(A) RNA enriched samples were subjected to cDNA synthesis using Illumina TruSeq preperation kit according to the manufacturer's protocol. Briefly, cDNA was synthesized by reverse transcriptase (Super-Script II) using poly-dT and random hexamer primers. The cDNA fragments were then blunt-ended through an end-repair reaction, followed by dA-tailing. Subsequently, specific double-stranded bar-coded adapters were ligated and library amplification for 15 cycles was performed. Pooled cDNA libraries, consisting of equal concentration bar-coded samples (wt and *Ercc1*$^{\Delta/+}$ samples), were sequenced in one lane each on the HiSeq2000 (Illumina).

qPCR

cDNA was synthesized with SuperScript First-Strand cDNA Synthesis Kit (Invitrogen) from 1µg of RNA. qPCR was performed on a C1000™ Thermal Cycler, CFX96 Real-Time System (Bio-Rad) using SYBR Green I (Invitrogen) and Platinum *Taq* polymerase (Invitrogen). Primer sequences were obtained from the PrimerBank PCR Primers database for Gene Expression Detection and Quantification (Spandidos et al., 2010) and are described in Supplementary table 4. Data were analysed using the second derivative maximum

method: ($E1_{\text{gene of interest}}^{\Delta CP\ (\text{cDNA of wt mice - cDNA of Ercc1}\Delta/-\ \text{mice})\ \text{gene of interest}}$)/($E_{house\ keeping}^{\Delta CP\ (\text{cDNA wt mice- cDNA of Ercc1}\Delta/-\ \text{mice})\ \text{housekeeping gene}}$). Data represent the average of values obtained from VMB from 4 animals ± the standard deviation. Expression was normalized against the average combined expression of two different housekeeping genes (B2m, Beta-2-microglobulin and Hprt1, Hypoxanthine-guanine phosphoribosyltransferase).

mRNA-Seq analysis

Reads were aligned to the mouse reference genome (mm9) using Tophat (version 1.3.1.Linux_x86_64, --coverage-search, -butterfly-search, --segment-mismatches 1,--segment-length 18) via the NARWHAL (Brouwer et al., 2012) automation software. SAMMate (http://sammate.sourceforge.net/) (Xu et al., 2011) was used to detect and quantify transcripts. Gene counts were calculated taking into account the reads mapped on exons or on exon-exon junctions. Differentially expressed genes were detected using the R package EdgeR (Robinson et al., 2010). An over-dispersed Poisson model was used to account for biological and technical variability. Genes with a false discovery rate (FDR) of < 0.05 and fold change ±1.5 were considered to be differentially expressed.

Human Parkinson's disease and incidental Lewy body disease datasets analysis

Microarray data from human substantia nigra (SN) were obtained from the GEO Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/). Three subseries from superseries GSE20186 (Zheng et al., 2010) were used in this analysis (GEO: GSE20186). Subseries GSE20159 consists of snap-frozen human SN of 16 individuals with a clinicopathological diagnosis of incidental Lewy body (ILB) disease; Lewy bodies were detected in the brainstem nuclei of locus coeruleus and SN, but not in higher cortical regions, consistently with Braak stage 3 criteria for Parkinson's disease (PD). This subseries also contained 17 age-, sex-, postmortem interval-, and RNA integrity number-matched controls. Subseries GSE20163 consists of SN samples from 8 PD subjects and 9 control subjects. Subseries GSE20164 consists of SNs samples from 6 PD and 5 control subjects obtained at autopsy.

Pathway analysis

Pathway enrichment analysis was conducted via two different approaches: Overrepresentation analysis (ORA) and Gene set enrichment analysis (GSEA).

ORA was performed in the Interactive pathway analysis (IPA) of complex genomics data software (Ingenuity Systems, www.ingenuity.com, Redwood City, CA) by employed a pre-filtered list of differentially expressed genes. The overrepresented canonical pathways were generated based on information in the Ingenuity Pathways Knowledge Base. A Fisher exact test was performed to determine the likelihood of obtaining at least the equivalent numbers of genes by as actually overlap between the input gene set and the genes present in each identified pathway.

GSEA was conducted on an unfiltered ranked list of genes. Genes in each dataset were ranked by the level of differential expression using a combination of Log2Fold change and the p-value as was proposed by Xiao (Chen et al., 2013).

$$\pi_i = \frac{|Fc_i|}{Fc_i} pi^{Fc_i}$$

Where:

Πi = Modified p-value of gene i

Fci = Log2 fold change of gene i

pi = p-value of gene i

Statistical significance of pathway enrichment score was ascertained by permutation testing over size-matched random gene sets, and multiple testing was controlled by false positives a family-wise error rate (FWER) threshold of 5% was used (Subramanian et al., 2005), this statistical is more conservative than FDR. Pathway information was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) available at the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). Because pathway databases are redundant, the R package Recipa was used to reduce the redundancy among KEGG pathways. Pathways with an overlapping higher than 75% in their genes were collapsed in one pathway (Vivar et al., 2013).

Comparison among *Ercc1*$^{\Delta/+}$, Parkinson's disease, and incidental Lewy body disease datasets

There were three datasets from two different organisms (human and mouse) and from three different platforms (Rna-Seq, Illumina and Affimetrix). To perform a fair comparison genes in each platform where filtered to analyze only the common genes in the three platforms.

The final lists of deregulated pathways were compared between the datasets. A hypergeometric distribution was used to determinate if the overlapping were higher than expected by chance. Additionally the factor of enrichment was calculated with the formula:

$EF=nAB/((nA\times nB)/nC)$

Where:

nA = Number of deregulated pathways in dataset A

nB= Number of deregulated pathways in dataset B

nC= Number of total pathways in KEGG database

nAB = Number of common deregulated pathways between A and B

Statistical analysis

Experiments were performed in four to eight independent biological replicates. Statistical analysis was performed with Prism4 (GraphPad Software). Comparison of multiple groups was performed by ANOVA or Kruskal-Wallis test, comparisons between two groups was performed by t-test.

Supplemental references

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