Supporting Information

Circulating (poly)phenol metabolites: neuroprotection in a 3D cell model of Parkinson’s disease

1. Supplementary data

1.1. Neuronspheroids monitoring along time

At different time points along culture progression, viability of differentiated neuronspheroids was assessed using a fluorescence microscopy-based method, the fluoresceine diacetate (FDA)/propidium iodide (PI) assay.\[^1\] Moreover, cell proliferation was also assessed by fluorescence microscopy using Click-iT Plus EdU AlexaFluor\textsuperscript{TM} 488 Assay kit (Life Technologies). Neuronspheroids sampling was performed along differentiation and neuronspheroids disrupted in 0.1 M citric acid with 1% Triton X-100, at 37 °C, overnight, and nuclei stained with 0.1 % crystal violet and counted in a Fuchs–Rosenthal haemocytometer chamber.\[^2\] A sample size of at least 1 mL of culture was used to reliably determine cell concentration, neuronspheroids counting and perform aggregate size measurements using ImageJ software.

1.2. Neuronspheroids synaptic activity

Evoked synaptic activity of Neuronspheroids was assessed by the method of Gaffield 2006,\[^3\] and already reported by us.\[^4\] LUHMES Neuronspheroids were collected at 0 and 7 days of differentiation and seeded in PLO-fibronectin pre-coated glass coverslips, and incubated with 100 mM KCl buffer (5 mM HEPES-NaOH, pH 7.4; 10 mM glucose; 2.5 mM calcium chloride; 1 mM magnesium chloride; 100 mM potassium chloride; 37 mM sodium chloride) for 5 min. Afterwards, 100 mM KCl buffer was removed and Neuronspheroids incubated with 10 μM FM-1-43 dye (Invitrogen) in 5 mM KCl buffer (5 mM HEPES-NaOH, pH 7.4; 10 mM glucose; 2.5 mM calcium chloride; 1 mM magnesium chloride; 5 mM potassium chloride; 37 mM sodium chloride) for 15 min. Neuronspheroids were washed for 1 min with 5 mM KCl buffer with ADAVASEP-7 (Sigma) followed by three washes with 5 mM KCl buffer. Exocytosis was stimulated with 100 mM KCl buffer and samples were visualized live using a fluorescence microscope (Leica DMI6000) in order to monitor the decrease in fluorescence intensity over time. Fluorescence intensity was measured using ImageJ software.
Spontaneous synaptic activity of LUHMES neurospheroids was assessed by their response in calcium assay. Neurospheroids with 7 days of differentiation were incubated with 1x Fluo-4 Direct calcium reagent (Invitrogen) for 30 min at 37 °C, 5% CO₂ and for 15 min at RT. Samples were then imaged live using spinning disk microscopy (Nikon Eclipse Ti-E, confocal scanner: Yokogawa CSU-x1). Fluorescence change over time is defined as \( \Delta F/F_0 = (F-F_0)/F_0 \), where F is the fluorescence at any time point, and F₀ the baseline fluorescence determined by baseline fitting across the whole movie for each cell using SparkMaster plugin of ImageJ software.

1.3. Immunofluorescence microscopy

Neurospheroids were collected at 7 days of differentiation and processed for immunofluorescence staining. Briefly, neurospheroids were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) solution in PBS with 4% (w/v) sucrose and processed directly for immunostaining. The primary antibodies used for cell characterization were anti-βIII-tubulin (βIII-tub, Millipore), anti-glia fibrillary acidic protein (GFAP, DAKO) and anti-tyrosine hydroxylase (TH, Santa Cruz Biotechnology). The secondary antibodies used were goat anti-mouse IgG-AlexaFluor 488 and goat anti-rabbit IgG AlexaFluor 594 (Life Technologies). Cell nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI, Life Technologies). Preparations were visualized using a point-scan confocal (Leica SP5) microscope. Merge between channels, maximum z-projections, and orthogonal projections, as well as linear brightness and contrast adjustment of the images were created using ImageJ software.

1.4. Western blot

Neurospheroids were lysed in TX-100 lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100 and 1x complete protease inhibitors cocktail (Roche)) and protein quantification was carried out using the Micro BCA™ Protein Assay kit (Thermo Fisher). Protein samples were subjected to a gel electrophoresis using a NuPAGE 4-12% Bis-Tris Gel with MES running buffer (Invitrogen) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% non-fat dried milk powder in 0.1% Tween-20 in PBS (blocking solution) for 1h, followed by incubation with primary antibodies overnight at 4 °C, diluted in blocking solution and then with secondary antibodies (horseradish peroxidase-conjugated, ECL anti-mouse IgG or anti-rabbit
IgG; GE Healthcare) diluted 1:5000 in blocking solution, for 2h at RT. Anti-α-tubulin or anti-GAPDH antibodies were used as control to confirm equal loading of total protein. Membranes were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized using a ChemiDocTM XRS+ System (BioRad). Primary antibodies used for protein detection were anti-βIII-tubulin (Millipore), anti-synaptophysin (Syn, Millipore), anti-GFAP (DAKO), anti-TH (Santa Cruz), anti-Nestin (Millipore), anti-glutamate transporter (GLT-1, Millipore) and anti-Pituitary homeobox-3 (Pitx3, Abcam).

Supplementary material

Table S1. List of primers used for RT-qPCR analysis.

| Gene    | Protein                                | Primers forward (top) and reverse (bottom) |
|---------|----------------------------------------|---------------------------------------------|
| RPL22   | Ribosomal protein L22 (housekeeping gene) | CACGAAGGAGGAGGAGTGAAGTG TGTGGCAACACACTGACATT |
| TUJ1    | βIII-tubulin                            | GGGCCCTTGGACATCTCTTCC CCTCGGTGTAGTGACCTTG   |
| TH      | Tyrosine hydroxylase                   | AGCCCTACCAAGACCCAGAGCG GCCTGTACGGGTCGAAATT |
| DAT     | Dopamine transporter                    | ACCTTCCTCCTGGTCCCTGTTT CCATAGAACCAGCCACT   |
| SYP     | Synaptophysin                           | TTTTGAGAGGGTGCATGAAATG GCTGAGTCACCTCGGCTT  |
| NES     | Nestin                                 | TAAGGAGAAAAGGGTGAGTG GCAAGAGATCCCTTTGAGCG  |
| GFAP    | Glial fibrillary acid protein           | AGAGGATCGAAGGCCAGGAG GGTACCCCAACACCCTACT   |
| ATF4    | Activating transcription factor 4       | GCTGGGCTGTGGAGGGTTGG CTCCGGACTAGGGGGCAA     |
| GSR     | Glutathione reductase                   | CGTTGGAGGTGCTGAAGTTTCTC TACGATCGTGATGACCT   |
| NQO1    | NAD(P)H quinone dehydrogenase 1         | TGACATATAGCATTGGGACAC ACATCTGTCCTTGATCTTT  |
| HSP40   | DnaJ (heat shock protein 40 kD)         | AGGAAAGCTAAAGGGGAGT AGGAGTTCTGGCAACCGAAG   |
| BCL-2   | B-cell lymphoma 2                       | ATCGCCTGTTGAGTGACTAGG CAGCCAGAGAAATCAAACAGAG |
| BAX     | BCL-2 associated X protein              | TGGAGCTGAGGAGGATGTGGA GAAATTGGCTGACAAACAGTG |
| BECN1   | Beclin-1                                | CGTTGAAAGAAGCAGCAAGAT GCTGCTCTGGCATACAGC   |
| MAP2K1  | Mitogen-Activated Protein Kinase Kinase 1 | AACTCTCGTCATACCTGTGG GATCTCTGCTTCTCCCTCAG   |
Table S2. RealTime Ready Custom panel information. Assay ID, gene symbol (H. sapiens), alias and description according to [https://configurator.realtimeready.roche.com/](https://configurator.realtimeready.roche.com/).

| Assay ID | Gene Symbol | Alias | Description |
|----------|-------------|-------|-------------|
| 138446   | ACSL3       | ACS3; FACL3; PRO2194 | acyl-CoA synthetase long-chain family member 3 [Source:HGNC Symbol;Acc:3570] |
| 127578   | ACSL4       | ACS4; FACL4; LACS4; MRX63; MRX68 | acyl-CoA synthetase long-chain family member 4 [Source:HGNC Symbol;Acc:3571] |
| 119501   | ACSL5       | ACS2; ACS5; FACL5 | acyl-CoA synthetase long-chain family member 5 [Source:HGNC Symbol;Acc:16526] |
| 100816   | AKT1        | AKT; MGC99656; PKB; PKB-ALPHA; PRKBA; RAC; RAC-ALPHA | v-akt murine thymoma viral oncogene homolog 1 [Source:HGNC Symbol;Acc:391] |
| 114661   | ATF4        | CREB-2; CREB2; TAXREB67; TXREB | activating transcription factor 4 (tax-responsive enhancer element B67) [Source:HGNC Symbol;Acc:786] |
| 115129   | ATF6        | ATF6A | activating transcription factor 6 [Source:HGNC Symbol;Acc:791] |
| 118103   | ATG12       | APG12; APG12L; FBR93; HAPG12 | ATG12 autophagy related 12 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:588] |
| 125999   | ATG5        | APG5; APG5-LIKE; APG5L; ASP; hAPG5 | ATG5 autophagy related 5 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:589] |
| 120541   | ATG7        | APG7-LIKE; APG7L; DKFZp434N0735; GSA7 | ATG7 autophagy related 7 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:16935] |
| 102998   | AXIN2       | AXIL; DKFZp781B0869; MGC10366; MGC126582 | axin 2 [Source:HGNC Symbol;Acc:904] |
| 142318   | BAX         | BCL2L4 | BCL2-associated X protein [Source:HGNC Symbol;Acc:959] |
| 142759   | BBC3        | FLJ42994; JFY1; PUMA | BCL2 binding component 3 [Source:HGNC Symbol;Acc:17868] |
| 100083   | BCL2        | Bcl-2 | B-cell CLL/lymphoma 2 [Source:HGNC Symbol;Acc:990] |
| 100085   | BCL2A1      | ACC-1; ACC-2; BCL2L5; BFL1; GRS; HBPA1 | BCL2-related protein A1 [Source:HGNC Symbol;Acc:991] |
| 100088   | BCL2L1      | Bcl-X; bcl-xL; BCL-XL/S; bcl-xS; BCL2L; BCLX; BCLXL; BCLXS; DKFZp781P2092 | BCL2-like 1 [Source:HGNC Symbol;Acc:992] |
| 100115   | BECN1       | ATG6; beclin1; VPS30 | beclin 1, autophagy related [Source:HGNC Symbol;Acc:1034] |
| 100122   | BID         | FP497; MGC15319; MGC42355 | BH3 interacting domain death agonist [Source:HGNC Symbol;Acc:1050] |
| 100135   | BIRC3       | AIP1; API2; c-IAP2; cIAP2; HAIP1; hiap-1; HIAP1; MALT2; MIHC; RNF49 | baculoviral IAP repeat-containing 3 [Source:HGNC Symbol;Acc:591] |
| 146274   | BMP2        | BMP2A | bone morphogenetic protein 2 [Source:HGNC Symbol;Acc:1069] |
| 146294   | BMP4        | BMP2B; BMP2B1; MCOPS6; OFC11; ZYME | bone morphogenetic protein 4 [Source:HGNC Symbol;Acc:1071] |
| 110980   | BTG2        | MGC126063; MGC126064; PC3; TIS21 | BTG family, member 2 [Source:HGNC Symbol;Acc:1131] |
|          | CALR        | cC1qR; CRT; FLJ26680; RO; SSA | calreticulin [Source:HGNC Symbol;Acc:1455] |
| Gene ID   | Gene Symbol   | Description                                                                 |
|----------|---------------|------------------------------------------------------------------------------|
| 113395   | CCL5          | chemokine (C-C motif) ligand 5                                               |
| 142502   | CCND1         | cyclin D1                                                                    |
| 101384   | CCND2         | cyclin D2                                                                    |
| 144097   | CDKN1A        | cyclin-dependent kinase inhibitor 1A (p21, Cip1)                              |
| 100855   | CDKN1B        | cyclin-dependent kinase inhibitor 1B (p27, Kip1)                              |
| -        | CEBPD         | CCAAT/enhancer binding protein (C/EBP), delta                                |
| 113740   | CPT2          | carnitine palmitoyltransferase 2                                              |
| 141077   | CSF1          | colony stimulating factor 1 (macrophage)                                    |
| 116795   | DAB2          | disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)          |
| 100355   | DDIT3         | DNA-damage-inducible transcript 3                                            |
| 115809   | DNAJC3        | DnaJ (Hsp40) homolog, subfamily C, member 3                                  |
| 143550   | EGFR          | epidermal growth factor receptor                                              |
| 148159   | EMP1          | epithelial membrane protein 1                                                |
| 103087   | FABP1         | fatty acid binding protein 1, liver                                           |
| 145362   | FAS           | Fas (TNF receptor superfamily, member 6)                                     |
| -        | FCER2         | Fc fragment of IgE, low affinity II, receptor for (CD23)                     |
| 104340   | FOSL1         | FOS-like antigen 1                                                           |
| 117090   | FTH1          | ferritin, heavy polypeptide 1                                                |
| 101471   | GADD45A       | growth arrest and DNA-damage-inducible, alpha                                |
| 115714   | GADD45B       | growth arrest and DNA-damage-inducible, beta                                 |
| 110864   | GATA3         | GATA binding protein 3                                                       |
| 147654   | GCLC          | glutamate-cysteine ligase, catalytic subunit                                 |
| 114136   | GCLM          | glutamate-cysteine ligase, modifier subunit                                  |
| 111427   | GSR           | glutathione reductase                                                        |
| 102005   | HERPUD1       | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 |

[Source:HGNC Symbol;Acc]
| Accession | Symbol | Description |
|-----------|--------|-------------|
| 142326 | HES1 | hairy and enhancer of split 1, (Drosophila) [Source:HGNC Symbol;Acc:5192] |
| - | HES5 | hairy and enhancer of split 5 (Drosophila) [Source:HGNC Symbol;Acc:19764] |
| 142325 | HEY1 | hairy/enhancer-of-split related with YRPW motif 1 [Source:HGNC Symbol;Acc:4880] |
| 112778 | HEY2 | hairy/enhancer-of-split related with YRPW motif 2 [Source:HGNC Symbol;Acc:4881] |
| 137073 | HEYL | hairy/enhancer-of-split related with YRPW motif-like [Source:HGNC Symbol;Acc:4882] |
| 110977 | HMOX1 | heat shock protein 90kDa alpha (cytosolic), class A member 2 [Source:HGNC Symbol;Acc:5256] |
| 100489 | HSP90B1 | heat shock protein 90kDa beta (Grp94), member 1 [Source:HGNC Symbol;Acc:12028] |
| 110730 | HSPA4 | heat shock 70kDa protein 4 [Source:HGNC Symbol;Acc:5237] |
| 110805 | HSPA5 | heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) [Source:HGNC Symbol;Acc:5238] |
| 100945 | ICAM1 | intercellular adhesion molecule 1 [Source:HGNC Symbol;Acc:5344] |
| 104631 | ID1 | inhibitor of DNA binding 1, dominant negative helix-loop-helix protein [Source:HGNC Symbol;Acc:5360] |
| 110609 | IFNG | interferon, gamma [Source:HGNC Symbol;Acc:5438] |
| 117683 | IFRD1 | interferon-related developmental regulator 1 [Source:HGNC Symbol;Acc:5456] |
| 144798 | IRF1 | interferon regulatory factor 1 [Source:HGNC Symbol;Acc:6116] |
| 108043 | JAG1 | jagged 1 [Source:HGNC Symbol;Acc:6188] |
| 112383 | LFNG | LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase [Source:HGNC Symbol;Acc:6560] |
| 146268 | LRG1 | leucine-rich alpha-2-glycoprotein 1 [Source:HGNC Symbol;Acc:29480] |
| 106176 | MAP3K1 | mitogen-activated protein kinase kinase kinase 1 [Source:HGNC Symbol;Acc:6848] |
| 102930 | MCL1 | myeloid cell leukemia sequence 1 (BCL2-related) [Source:HGNC Symbol;Acc:6943] |
| 104396 | MMP7 | matrix metallopeptidase 7 (matrilysin, uterine) [Source:HGNC Symbol;Acc:7174] |
| 100977 | MYC | v-myc myelocytomatosis viral oncogene homolog (avian) [Source:HGNC Symbol;Acc:7553] |
| 142322 | NOTCH1 | notch 1 [Source:HGNC Symbol;Acc:7881] |
| Gene ID  | Gene Name          | Description                                                                 | Source                        |
|---------|--------------------|------------------------------------------------------------------------------|-------------------------------|
| 147227  | NQO1               | NAD(P)H dehydrogenase, quinone 1                                            | [Source:HGNC Symbol;Acc:2874] |
| 113212  | OLR1               | oxidized low density lipoprotein (lectin-like) receptor 1                    | [Source:HGNC Symbol;Acc:8133] |
| 101524  | PCNA               | PCNA antisen RNA (non-protein coding)                                        | [Source:HGNC Symbol;Acc:37184]|
| 104411  | PPARD              | peroxisome proliferator-activated receptor delta                             | [Source:HGNC Symbol;Acc:9235] |
| 111252  | PTCH1              | patched 1                                                                   | [Source:HGNC Symbol;Acc:9585] |
| 105606  | RAF1               | v-raf-1 murine leukemia viral oncogene homolog 1                             | [Source:HGNC Symbol;Acc:9829] |
| 101596  | RB1                | retinoblastoma 1                                                             | [Source:HGNC Symbol;Acc:9884] |
| 117069  | SLC27A4            | solute carrier family 27 (fatty acid transporter), member 4                  | [Source:HGNC Symbol;Acc:10998]|
| 101122  | SOCS3              | suppressor of cytokine signaling 3                                           | [Source:HGNC Symbol;Acc:19391]|
| 119555  | SORBS1             | sorbin and SH3 domain containing 1                                           | [Source:HGNC Symbol;Acc:14565]|
| 113560  | SQSTM1             | sequestosome 1                                                               | [Source:HGNC Symbol;Acc:11280]|
| 101180  | STAT1              | signal transducer and activator of transcription 1, 91kDa                    | [Source:HGNC Symbol;Acc:11362]|
| 147880  | TNF                | Tumor necrosis factor Precursor (TNF-alpha)(Tumor necrosis factor ligand superfamily member 2)(TNF-a)(Cachectin) [Contains Tumor necrosis factor, membrane form;Tumor necrosis factor, soluble form] | [Source:UniProtKB/SwissProt;Acc:P01375]|
| 101266  | TNFSF10            | tumor necrosis factor (ligand) superfamily, member 10                        | [Source:HGNC Symbol;Acc:11925]|
| 116591  | TXNL4B             | thioredoxin-like 4B                                                          | [Source:HGNC Symbol;Acc:26041]|
| 114835  | TXNRD1             | thioredoxin reductase 1                                                      | [Source:HGNC Symbol;Acc:12437]|
| 109914  | ULK1               | unc-51-like kinase 1 (C. elegans)                                            | [Source:HGNC Symbol;Acc:12558]|
| 146965  | WISP1              | WNT1 inducible signaling pathway protein 1                                   | [Source:HGNC Symbol;Acc:12769]|
| 104468  | WNT1               | wingless-type MMTV integration site family, member 1                         | [Source:HGNC Symbol;Acc:12774]|
| 104488  | WNT2B              | wingless-type MMTV integration site family, member 2B                        | [Source:HGNC Symbol;Acc:12781]|
| 104494  | WNT3A              | wingless-type MMTV integration site family, member 3A                        | [Source:HGNC Symbol;Acc:15983]|
| 146266  | WNT5A              | wingless-type MMTV integration site family, member 5A                        | [Source:HGNC Symbol;Acc:12784]|

Note: Source:HGNC Symbol;Acc:nnnnn indicates the HGNC Symbol and Accession number for each gene.
| ID   | Symbol | Description                                      | Source                      |
|------|--------|--------------------------------------------------|-----------------------------|
| 47821| WNT6   | wingless-type MMTV integration site family, member 6 | [Source:HGNC Symbol;Acc:12785] |
| 102065| B2M    | beta-2-microglobulin                             | [Source:HGNC Symbol;Acc:914] |
| 143636| ACTB   | actin, beta                                      | [Source:HGNC Symbol;Acc:132] |
| 141139| GAPDH  | glyceraldehyde-3-phosphate dehydrogenase         | [Source:HGNC Symbol;Acc:4141] |
Figure S1. LUHMES neurospheroids cell culture characterization along time. Cells were inoculated in stirred suspension culture systems and aggregated (Agg) for 2 days. Dopaminergic differentiation was induced by removing bFGF from medium and by introduction of human GDNF, cAMP and tetracycline for 14 days (Differentiation Medium). 

(A) Representative images of the culture status at the indicated differentiation (Diff) days: fluorescent live/dead assay using fluorescein diacetate (FDA) for identification of live cells (green) and propidium iodide (PI) for identification of dead cells (red). Neurospheroids diameter (μm) profile and neurospheroids concentration along differentiation. 

(B) Representative images of neurospheroids proliferation status at the indicated differentiation (Diff) days and percentage of proliferative cells (EdU positive cells): EdU labeling of proliferative cells (green) and nuclei counterstain with DAPI (blue). Data are mean ± SD of three independent cultures. Scale bar: 100 μm.
Figure S2. LUHMES neurospheroids culture neural population characterization. (A) RT-qPCR analysis of Nestin, βIII-tubulin, synaptophysin, TH and DAT gene expression; fold increase in gene expression of neurospheroids along culture time normalized for 0d of differentiation. Statistical differences are donated as ***p<0.001, **p<0.01 and *p<0.05 relative to 0d differentiation. (B) Detection of βIII-tubulin, synaptophysin, TH, glial fibrillary acidic protein (GFAP), glutamate transporter (GLT-1), pituitary homeobox-3 (Pitx3) and Nestin by Western blot analysis and corresponding fold change in protein levels obtained by densitometry normalized for 0d of differentiation; α-tubulin was used as loading control. Statistical differences are donated as *p<0.05, **p<0.01, ***p<0.001 relative to 0d differentiation, by two-way ANOVA analysis with Tukey’s post multiple comparison test. (C) Immunofluorescence microscopy of neurospheroids (7d Diff); scale bar: 20 μm; βIII-tubulin (green), TH (green), GFAP (red), and DAPI (blue).
Figure S3. Differentiated LUHMES neurospheroids (7d Diff) were submitted to MPP⁺. (A) Dose-response curve of 0.5-100 μM MPP⁺ induced lesion for 24 hours with respective IC50, determined by Presto blue assay. (B) RT-qPCR analysis of βIII-tubulin, TH and DAT gene expression; fold change in gene expression of neurospheroids treated with 5 μM MPP⁺ after 6h, 24h and 48h. Statistical differences are denoted as *p<0.05 or ***p<0.0001 as indicated, by two-way ANOVA analysis with Tukey’s post multiple comparison test. (C) Detection of βIII-tubulin and TH by western blot analysis and corresponding fold change in protein levels obtained by densitometry and normalized for control condition (no MPP⁺). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are mean ± SD of three independent cultures.
Figure S4. Venn diagram based on comparative toxicogenomic database showing the association of all the differentially expressed genes for cat-sulf (A) and pyr-sulf (B) treatments with neurodegenerative diseases and in particular with PD.
Supplementary references

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