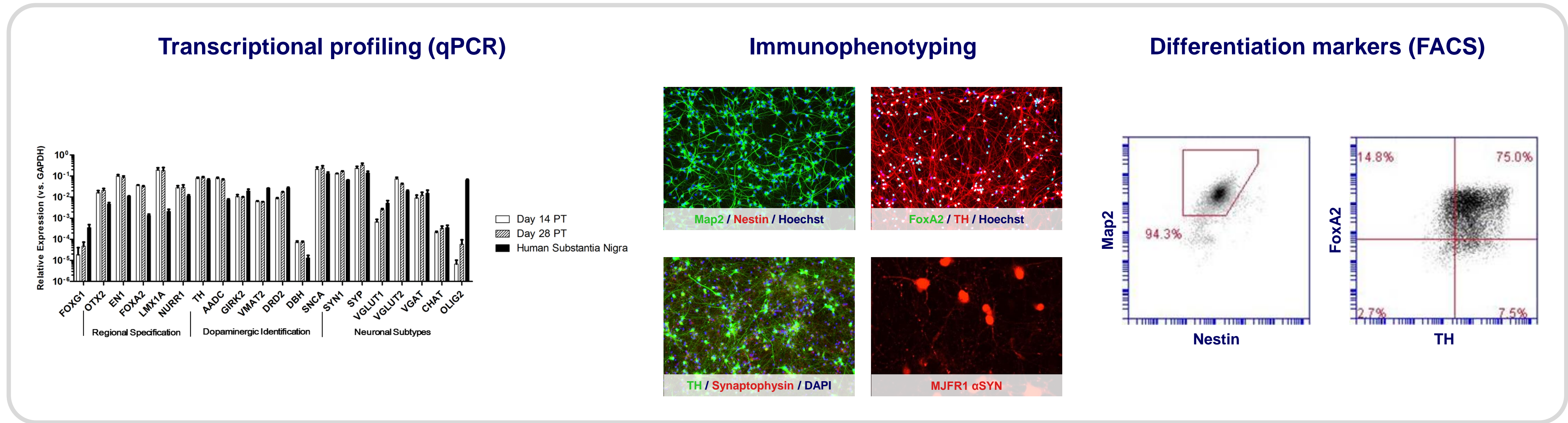


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**Aim of the study:** set up a compound-rescue assay in a physiologically relevant cell system to screen for novel neuroprotective drugs

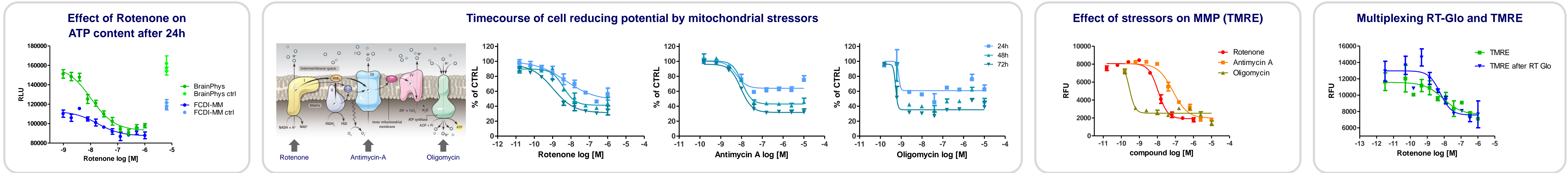
**iCell®DopaNeurons characterization provided by FCDI**

iCell®DopaNeurons are a fully differentiated, highly pure population of human midbrain dopaminergic neuron derived using FCDI's proprietary differentiation and purification protocols.



## Rotenone-induced mitochondrial toxicity

Mitochondrial dysfunction and oxidative stress are pathophysiological mechanisms implicated in many neurodegenerative experimental models<sup>1</sup>. Different molecules impairing the mitochondrial membrane potential (MMP) were tested to check their toxic effect on iCell®DopaNeurons viability: Antimycin A, that inhibits mitochondrial respiration complex III; Oligomycin as a complex V inhibitor and Rotenone, a complex I inhibitor.



CellTiter-Glo® (ATP-dependent assay) was used to measure the effect of Rotenone insult initially after 24h in both culture conditions (FCDI-MM or BrainPhys™) showing no difference in the overall potency of the stressor, but a better signal window using BrainPhys™.

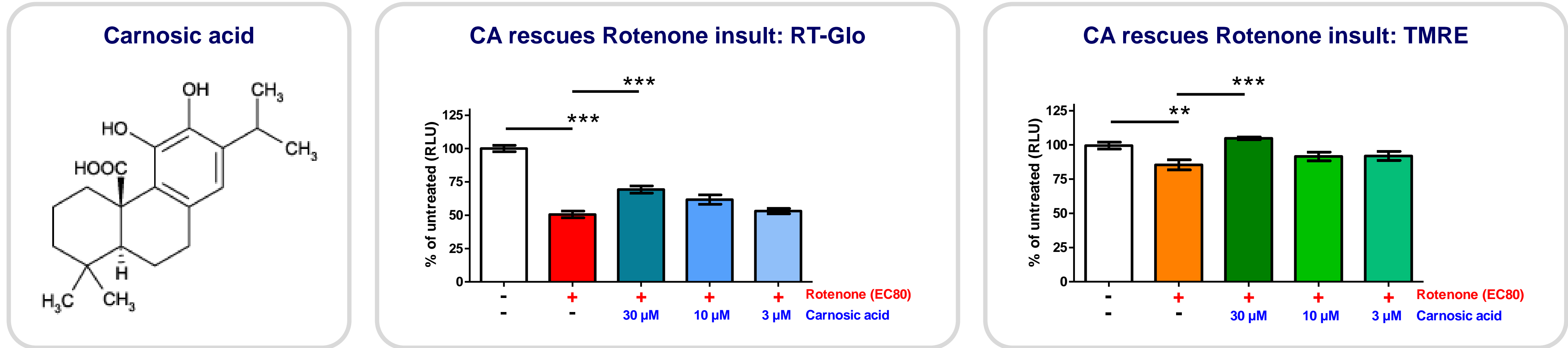
RealTime-Glo™ MT Cell Viability Assay (RT-Glo) is an ATP-independent nonlytic assay that can be measured in real time and continuous manner and correlates with viable cells. Rotenone insult was measured also using RT-Glo generating results comparable with CellTiter-Glo®. Rotenone reduced the cell viability in a time-dependent manner with  $EC_{50}$  2.3 nM at both 24 and 48h, but with a maximal effect at 48h. Moreover, a comparison using Oligomycin and Antimycin A was performed to confirm the Rotenone injury at different timepoints and to ensure that a maximal reduction of the cell viability occurred.

TMRE, a cell-permeant fluorescent dye that is sequestered by active mitochondria was analysed. Using this readout Rotenone reduced the MMP with an  $EC_{50}$  of 9.5 nM.

RT-Glo can be multiplexed ensuring more than one readout within the same plate. After an initial segmentation using calcein >4,000, the Rotenone insult was measured after 48h, monitoring TMRE.

## Neuroprotection by Carnosic acid (CA)

Carnosic acid is a natural compound found in rosemary and sage that easily crosses the blood-brain-barrier. CA is pro-electrophile agent has been shown to be a neuroprotective agent against oxidant/stressful conditions both *in vitro* and *in vivo* experimental models<sup>2,3</sup>.

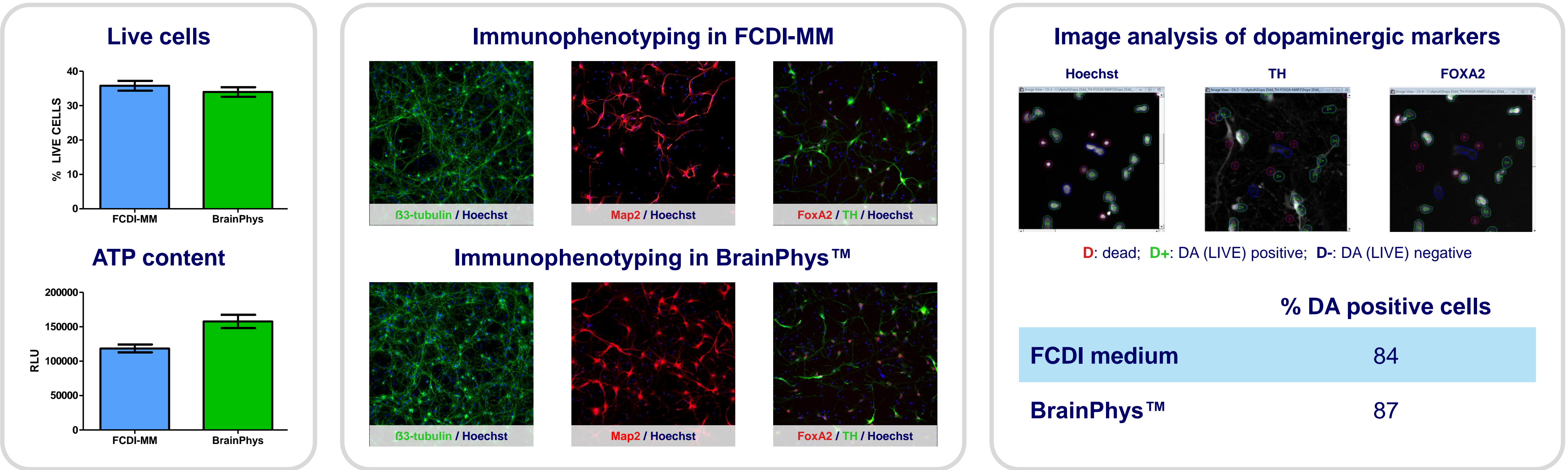


One hour before injury, carnosic acid (30, 10 or 3  $\mu\text{M}$ ) was added and then an EC<sub>80</sub> of Rotenone was subsequently applied. Viability was measured at 48h using RT-Glo. Mean $\pm$ SEM is shown (n $\geq$ 6). Replicates in 2 independent experiments.

One hour before injury, carnosic acid (30, 10 or 3  $\mu$ M) was added and then an EC<sub>80</sub> of Rotenone was applied. MMP was monitored at 48h using TMRE showing results comparable to RT-Glo. Mean $\pm$ SEM is shown (n $\geq$ 6). Replicates in 2 experiments.

## Comparison of culture in BrainPhys™ vs FCDI's maturation medium

BrainPhys™ Neuronal Medium is a serum-free, low-glucose medium described for culture and maturation of hPSC-derived neurons. FCDI's maturation medium (FCDI-MM) has a 10X concentration of glucose compared to BrainPhys™, which could potentially make cells less sensitive to a toxic insult.



Cell<sup>®</sup>DopaNeurons were plated and after 7 days the FCDI differentiation medium was either maintained or switched to BrainPhys<sup>™</sup> for further 7 days. Total ATP content was measured using CellTiter-Glo<sup>®</sup> (left panel, bottom); cells were also stained with various neuronal markers and analysed. Live cells were defined as having a nuclear area >30  $\mu$ m (left panel); Hoechst,  $\beta$ 3-tubulin, MAP2, FOXA2 and TH positive cells were analysed in both culture condition (central panel); the masks used for the analysis and obtained percentage of cells expressing dopaminergic (DA) markers is reported in the right panel.

## Conclusions

iCell®DopaNeurons represent a suitable model to further investigate the neurotoxicity mechanism of Rotenone injury and to screen compounds with neuroprotective potential for neurodegenerative diseases, using CA as a reference neuroprotectant compound. For drug discovery purposes a high-throughput phenotypic assay using TMRE can be multiplexed with a viability readout RT-Glo.

## Perspectives

- complete the assay set-up (Z factor)
- screening of LOPAC®1280
- further characterization of positive hits
- assay set-up using cells with disease-linked mutations

## References

- <sup>1</sup> von Wrangel C *et al.*, 2015. The rotenone-induced rat model of Parkinson's disease: behavioural and electrophysiological findings. *Behavioural Brain Research*; 279:52-61.
- <sup>2</sup> Satoh T *et al.*, 2008. Carnosic acid, a catechol-type electrophilic compound, protects neurons both *in vitro* and *in vivo* through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1. *Journal of Neurochemistry*; 104(4):1116-31.
- <sup>3</sup> Zhang D *et al.*, 2015. Protection from cyanide-induced brain injury by the Nrf2 transcriptional activator carnosic acid. *J Neurochem* 133(6): 898–908.

## Materials and methods

Cells were thawed following manufacturer's instructions for use and recommended practices.

About 15,000 viable cells/well were plated in 384 Greiner PDL-plates, previously coated one hour with polyornithine and overnight with laminin (both from Sigma Aldrich).

During culture, half of the medium was replaced twice a week.

After 7 days in FCDI-MM when not differently specified the medium was replaced by BrainPhys™ supplemented with iCell Neurons Medium Supplement and iCell Nervous System Supplement (both from FCDI), N2 (Thermo Fisher Scientific), laminin and P/S as suggested by FCDI on the application protocol for iCell®DopaNeurons for Maestro multielectrode array (MEA).

All the experiments were performed after at least 14 days in culture. The day of the experiment a complete replacement of the medium before the stressor/neuroprotectant treatment was performed.

All the stressors and carnosic acid (purchased from Sigma Aldrich) were freshly prepared, dissolved DMSO and added using Echo dispenser.

Calcein, TMRE and Hoechst were purchased from Thermo Fisher Scientific; antibodies: MAP2 from Sigma Aldrich, FOXA2 from Cell Signalling,  $\beta$ -3-tubulin and TH were from Abcam.

CellTiter-Glo and RT-Glo luminescence were measured using CLARIOstar (BMG LABTECH) or Envision (Perkin Elmer) while Calcein, TMRE and all the immunostaining were measured and analysed using the iCell Analyzer 2200 (GE Healthcare Life Sciences).