A LUHMES 3D dopaminergic neuronal model for neurotoxicity testing allowing long-term exposure and cellular resilience analysis

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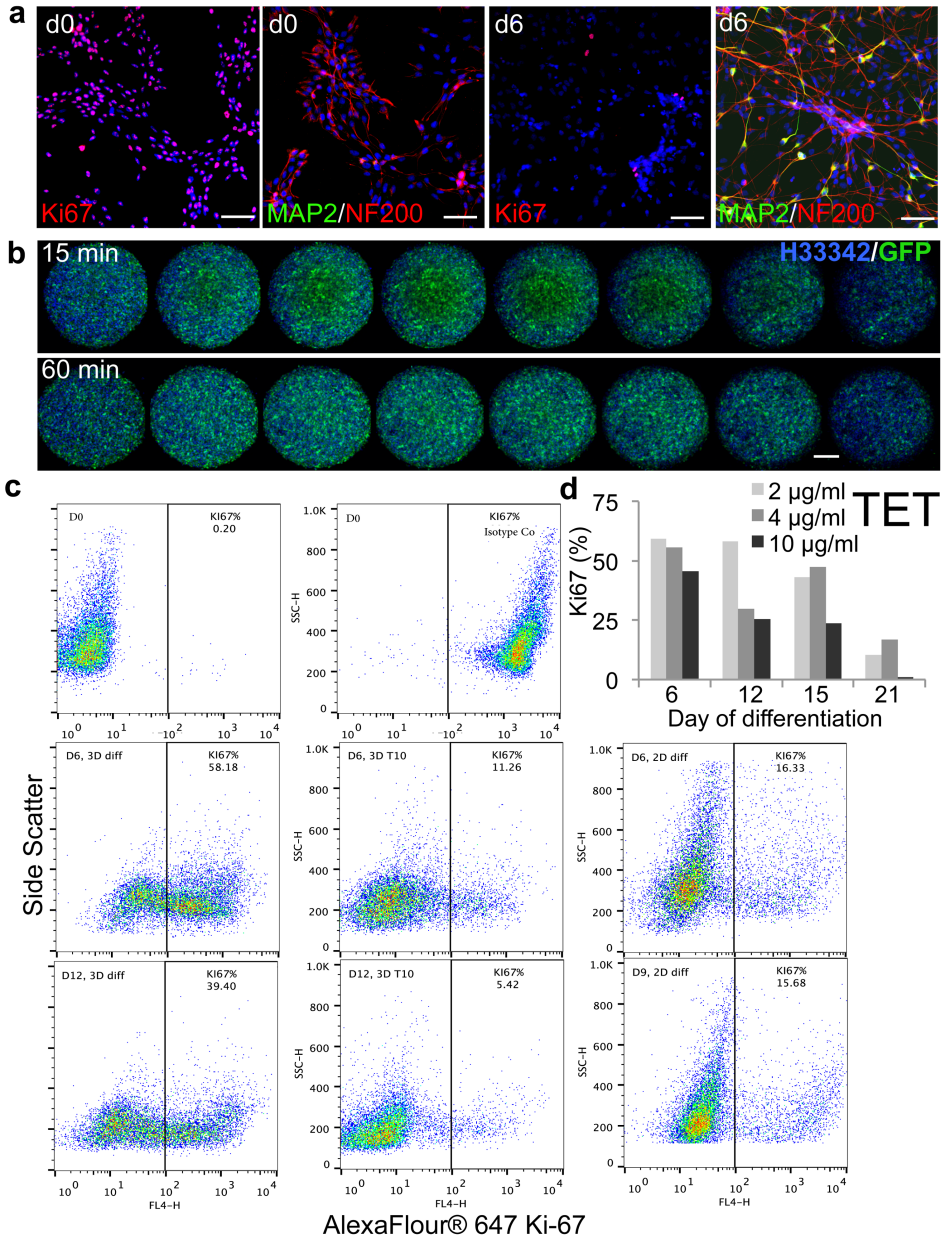
Supplementary material

Supplementary Table S1. SYBR Green PCR primer sequences.

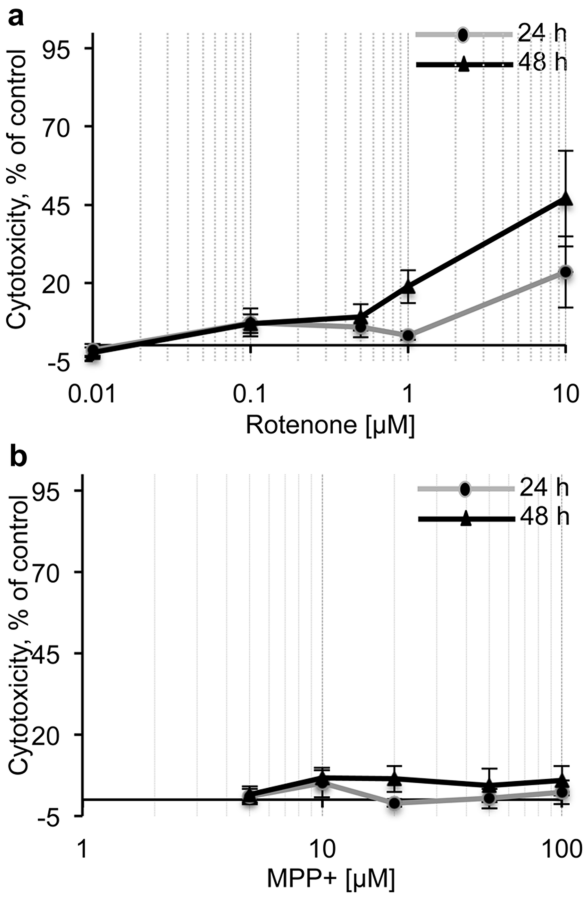
Name	Forward sequence, 5‘-3‘	Reverse Sequence, 5‘-3‘
ASS1	TGCTCCCTGGAGGATGCCTG	GTGTAGAGACCTGGAGGCGC
ATF4	GGCTGGCTGTGGATGGGTTG	CTCCTGGACTAGGGGGGCAA
CBS	TCCTGGGAATGGTGACGCTT	GTGCTGTGGTACTGGATCTG
CTH	TGGATGATGTGTATGGAGGTACAAACAGG	GCCTTCAATGTCAATCACCTTCTGGG
GAPDH	CACCATCTTCCAGGAGCGAGATC	GCAGGAGGCATTGCTGATGATC
MLF1IP	TTTGTAAGGCAGCCATCGCC	CTGTGGCTCTAACCGAAGCA
SHMT2	CAACCTGGCACTGACTGCTC	GATGTCCGCGTGCTTGAAAG
TYMS	CAGCTTCAGCGAGAACCCAG	ACCTCGGCATCCAGCCCAAC

Supplementary Table S 2. p values for statistical significant changes in neural gene expression upon induction of differentiation in 3D and 2D cultures: one-way ANOVA test followed by Dunnett's post-hoc test. p-value <0.05 is denoted in the table by *, p<0.01 by **, and p<0.001 by ***, respectively

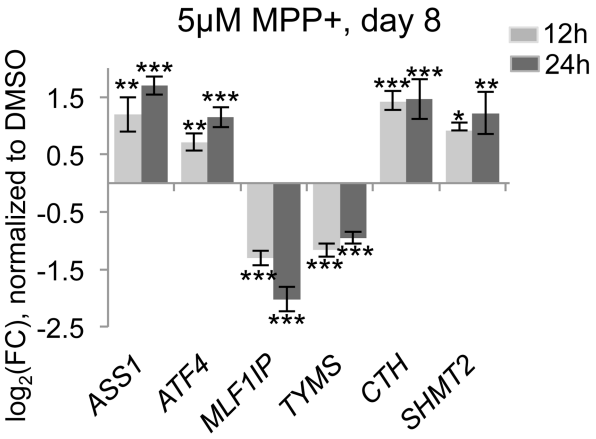
	3D + T10 differentiation						2D differentiation		
genes \ days	3	6	9	12	15	21	3	6	9
TH	***	***	***	***	***	***	***	***	***
Syn1	***	***	***	***	***	***	***	***	***
VMAT2	n.s.	***	***	***	***	***	n.s.	**	***
NeuN	**	***	***	***	***	***	***	***	***
DAT	*	**	*	n.s.	n.s.	*	*	***	***
β-III-tub	***	***	***	***	***	***	n.s.	**	**
Nestin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	*
Ki67	n.s.	*	**	**	***	**	n.s.	n.s.	n.s.



Supplementary Figure S1 **a** Two first photographs show undifferentiated LUHMES expressing Ki-67, neurofilament (NF200), but not MAP2. Two further photographs in panel **a** show changes in morphology upon induction of differentiation in 2D, induction of MAP2 expression, and reduction of Ki-67 expression. *Scale* bar is 50 µm **b** Penetration assay with Hoechst 33342: 12-day-old aggregates of GFP-expressing LUHMES were differentiated according to 3D diff protocol and were stained with Hoechst 33342. Montages of confocal optical slices of the aggregates after 15 and 60 min incubation with Hoechst 33342 are shown to demonstrate time-dependent penetration of Hoechst 33342 through the aggregates. *Scale* bar is 100 µm. **c** Proliferation rate in course of differentiation in 2D, 3D diff and 3D+T10 treatment based on Ki-67 expression, measured by flow cytometry. Representative dot plots (Side Scatter vs. Fluorescence 4 (Alexa-647)) for both 3D conditions are shown for days 6 and 12 and for 2D cultures for days 6 and 9. Ki-67 expression in undifferentiated LUHMES (d0) was more than 99% and was used together with isotype control antibody staining to set the gates. **d** Effects of increased tetracycline concentration on Ki-67 expression in neuronal-differentiated LUHMES in 3D. LUHMES were differentiated following 3D diff protocol in differentiation medium supplemented with 2, 4 or 8 µg/ml tetracycline. Ki-67 expression was analyzed on day 6, 12, 15 and 21 after induction of differentiation by flow cytometry



Supplementary Figure S 2 Analysis of membrane integrity in LUHMES aggregates after exposure to rotenone and MPP$^+$. LUHMES cells were differentiated following 3D+T10 protocol and exposed reversely to different rotenone (**a**) and MPP$^+$ (**b**) concentrations from day 6 to day 8 (48 h) and from day 7 to day 8 (24 h). Cytotoxicity was analyzed using LDH release assay and is presented in % of positive (1% Triton X100) controls in four independent experiments (n = 4, mean ± SEM)



Supplementary Figure S 3 Perturbation of expression of the genes involved in C1 metabolism, oxidative stress, and DNA repair by 5 µM MPP+. ASS1, argininosuccinate synthase, AT4, activating transcription factor 4, CTH, cystathionase (cystathionine γ-lyase), MLF1IP, centromere protein U (MLF1 interacting protein), SHMT2,

Serine hydroxymethyl-transferase, and TYMS, thymidylate synthetase. The data are means of log2 (fold change) ± SEM of three independent experiments (9 technical replicates). (n=3, *$p<0.05$, **$p<0.01$, and ***$p<0.001$, one-way-ANOVA followed by Dunnett's post hoc test)