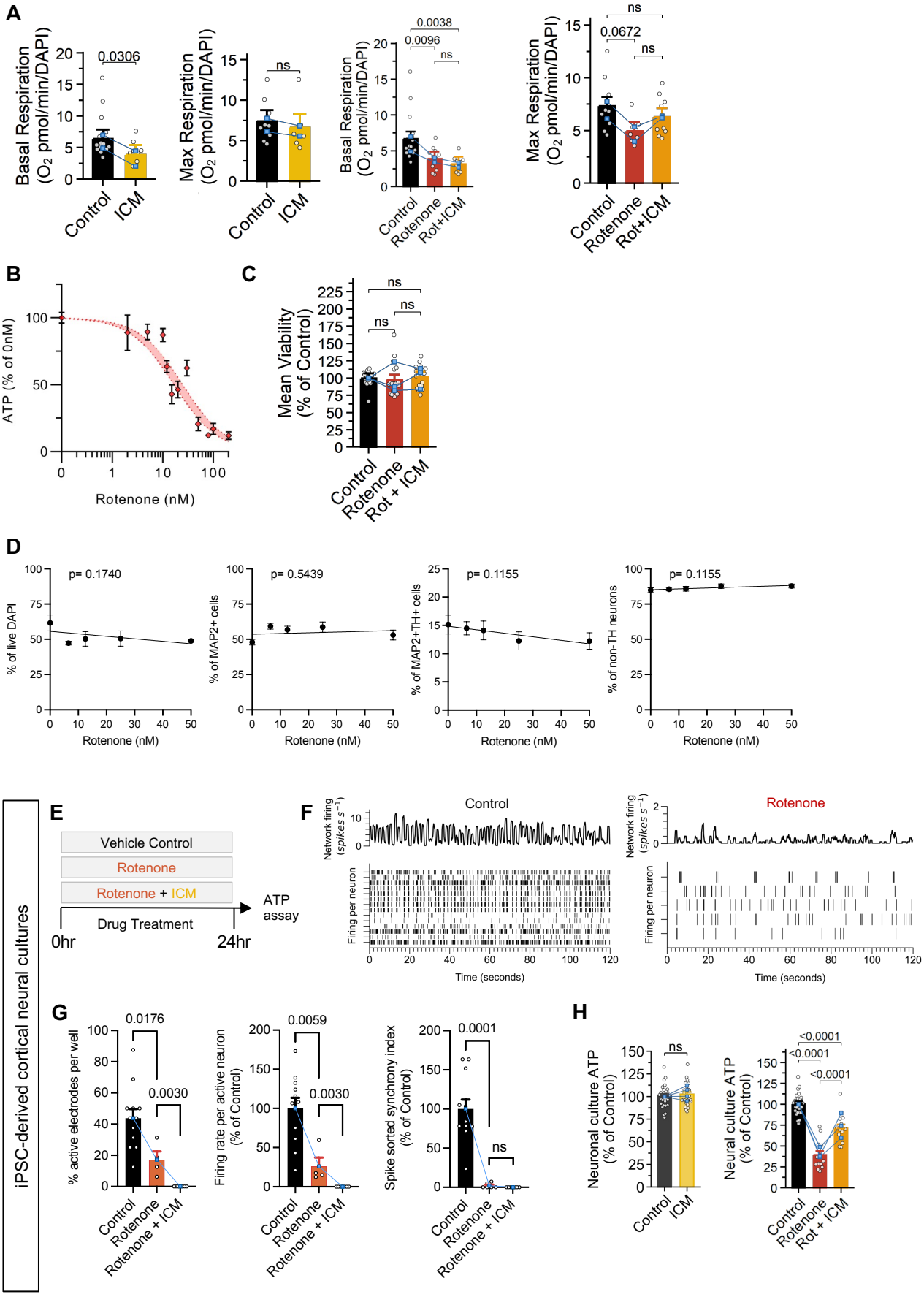
A
Basal Respiration (O2 pmol/min/DAPI)
0.0306
Control
ICM
Max Respiration (O2 pmol/min/DAPI)
ns
Control
ICM
Basal Respiration (O2 pmol/min/DAPI)
0.0038
0.0096
ns
Control
Rotenone
Rot+ICM
Max Respiration (O2 pmol/min/DAPI)
ns
0.0672
ns
Control
Rotenone
Rot+ICM
B
ATP (% of 0nM)
Rotenone (nM)
C
Mean Viability (% of Control)
ns
ns
ns
Control
Rotenone
Rot + ICM
D
% of live DAPI
p= 0.1740
% of MAP2+ cells
p= 0.5439
% of MAP2+TH+ cells
p= 0.1155
% of non-TH neurons
p= 0.1155
Rotenone (nM)
iPSC-derived cortical neural cultures
E
Vehicle Control
Rotenone
Rotenone + ICM
Drug Treatment
0hr
24hr
ATP assay
F
Control
Rotenone
Network firing (spikes s−1)
Firing per neuron
Time (seconds)
G
% active electrodes per well
0.0176
0.0030
Firing rate per active neuron (% of Control)
0.0059
0.0030
Spike sorted synchrony index (% of Control)
0.0001
ns
Control
Rotenone
Rotenone + ICM
H
Neuronal culture ATP (% of Control)
ns
Control
ICM
Neural culture ATP (% of Control)
<0.0001
<0.0001
<0.0001
Control
Rotenone
Rot + ICM

Figure S1 – Additional measures of metabolic effects from acute Rotenone and ICM exposure, related to Figure 2.

(A) Seahorse XF measurements of culture bioenergetics after 24-hour exposure to vehicle or ICMs by Seahorse XF MitoStress Test and Real Time ATP Rate assays. Rotenone concentrations used, as in Figure 2, at doses lowering ATP levels by ~50%. Bars represent means + S.E.M. **(B)** Intracellular ATP levels from cultures exposed to various concentrations of rotenone for 24 hours. [Inhibitor] vs. normalised response least squares fit was applied to the data in GraphPad Prism; the shaded area indicates the 95% confidence interval. Each data point is the mean of 3 - 13 wells across four experiments from four independent neural inductions; error bars indicate ± S.E.M. **(C)** Culture viability determined by flow cytometry analysis gating for DAPI- cells. Bars represent the mean + S.E.M. Data were normalised to the mean of the vehicle control. **(D)** Viability of neural cultures following rotenone exposure. Each point represents mean ± S.E.M of 3-5 replicate wells across one independent experiment. Statistical analysis and line of best fit determined via simple linear regression. **(E)** Schematic depicting treatment timelines for the maturation and drug exposure of neural cultures for analysis. **(F)** Multi-electrode array (MEA) extracellular recordings of electrophysiological activity. 120 second raster plots depict the action potential firing of individual neurons. Associated line graphs indicate firing density within synchronous network events (arbitrary units). Each plot depicts a single well. Each row depicts a single neuron. Each vertical line depicts an action potential. **(G)** Histograms summarising the effect of rotenone and ICM exposure on the firing rate of active neurons (Hz), number of active (>1 spike per minute) electrodes per well, and synchrony index per well. **(H)** ATP levels measured from the lysates of iPSC cortical-patterned cultures exposed to vehicle control, rotenone, or co-exposure with rotenone and ICMs for 24 hours. Bars represent the mean + S.E.M. (n = number wells, data from 3 independent experiments). **(A,C,G,H)** Data were normalised to the mean of the vehicle control. White circles represent individual well replicates, blue squares with blue paired lines represent averages per neural differentiation. **(A-C,H)** Statistical significance was determined using linear mixed-effects models, as described in the Methods. **(G)** Statistical significance determined by Mann-Whitney U-test on individual well replicates.

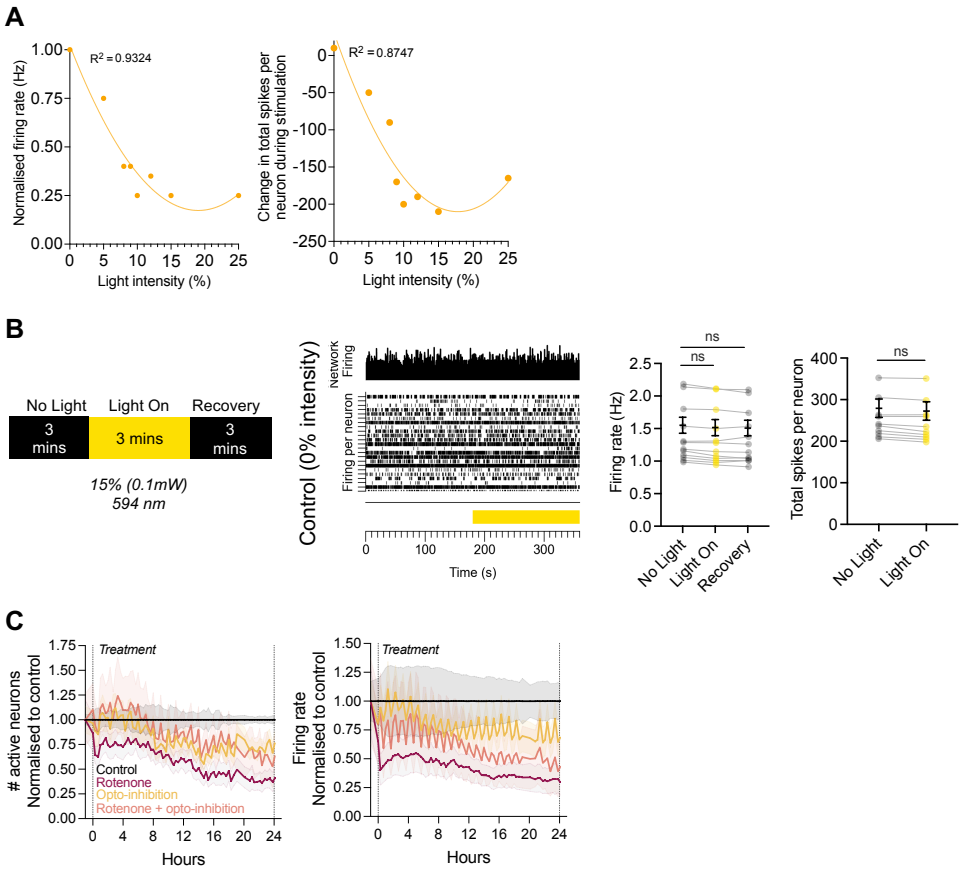

Figure S2 – Optogenetic inhibition attenuates loss of firing activity caused by rotenone exposure, related to Figure 3.

(A) Firing rate and action potential (spike) counts of halorhodopsin-expressing neurons following application of 594nm (yellow) light at various intensities. **(B)** Experimental paradigm of 3-minute-on 3-minute-off repeating pattern lasting 9 minutes. 360 second raster plots depict the action potential firing of individual neurons expressing halorhodopsin with 180s of optical stimulation at 0% intensity (indicated by the yellow bar) at 594 nm. Line graphs present various parameters of electrophysiological function (firing rate of active neurons, total spikes per neuron) pre-, during (evoked), and post-light stimulation for individual wells (each line is one well, n = 12 wells). Error bars indicate the means ± S.E.M. Statistical significance determined by Mann-Whitney U-test on individual well replicates. **(C)** Number of active neurons and active neuron firing rates before and during exposure to rotenone and/or opto-inhibition for 24-hours. Lines represent the mean change from pre-treatment, normalised to the control. Shaded areas depict ± S.E.M. n = 12 wells.

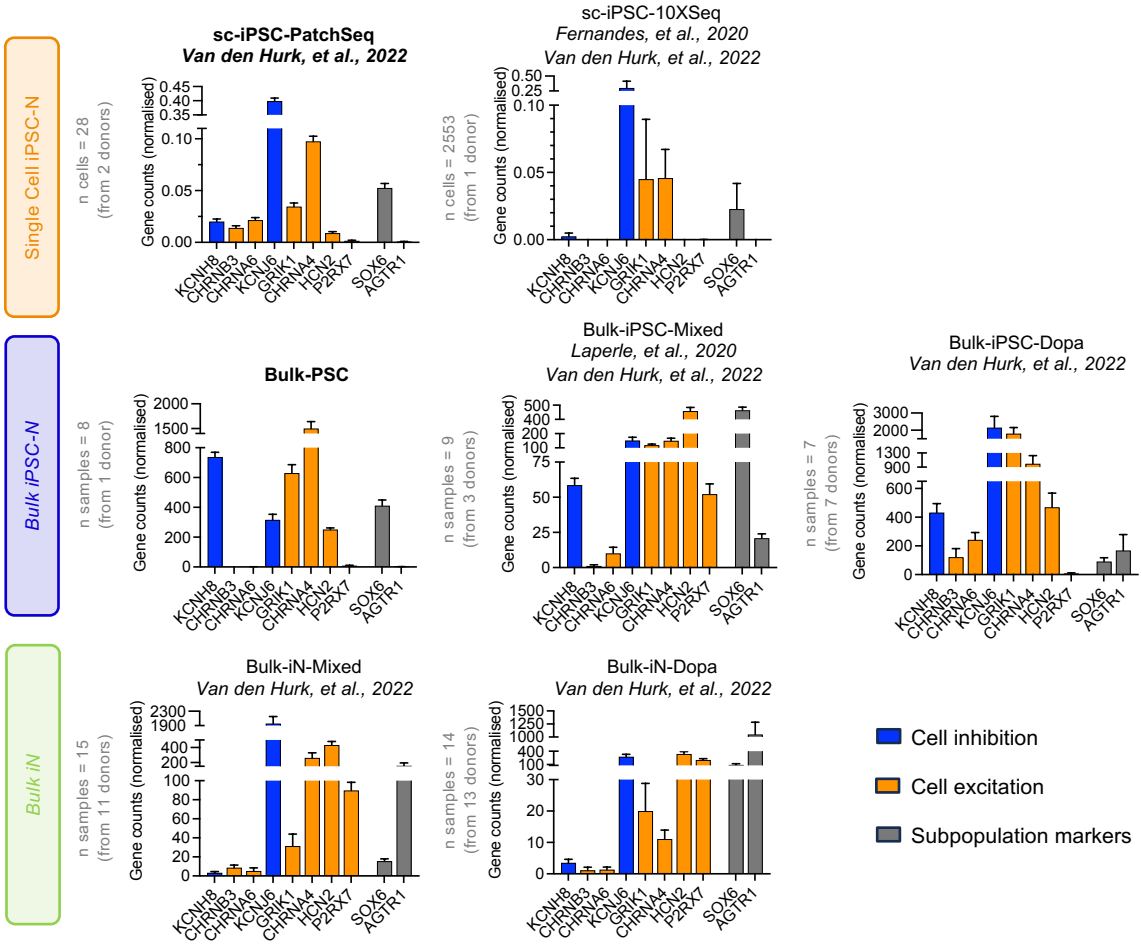

Figure S3 – Expression of chosen target genes in healthy midbrain iPSC and H9 cultures from bulk or single cell RNA-sequencing, related to Figure 4.

Top row shows single-cell RNA-sequencing analysis of iPSC-derived neurons, middle row shows bulk RNA-sequencing analysis for either H9 or iPSC-derived neurons and bottom row shows bulk RNA-sequencing for induced neurons. Titles in bold represent single-cell or bulk RNA-sequencing on our in-house midbrain neuronal cultures.

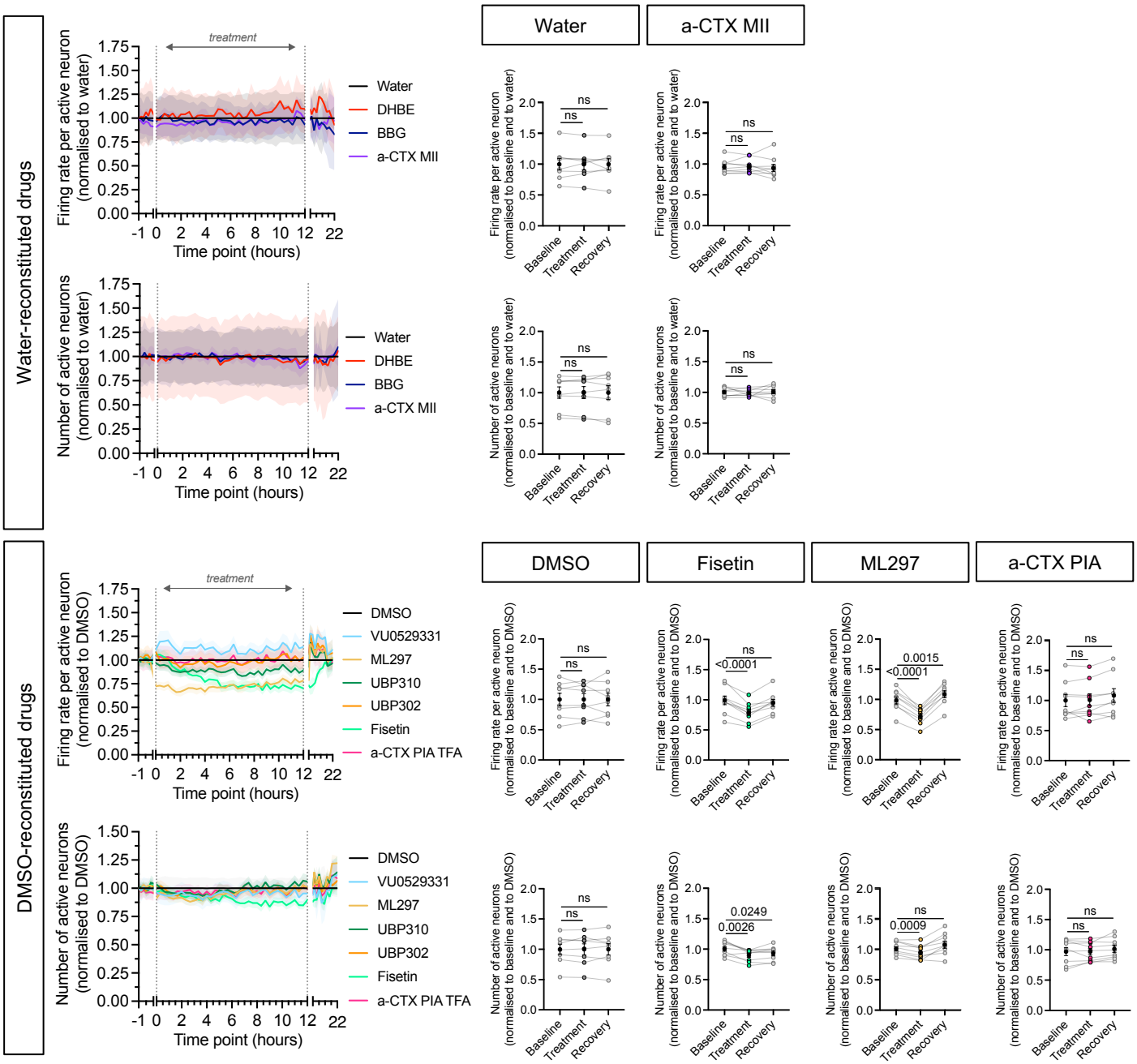

Figure S4 – Effects of SN-ICM candidate compounds on midbrain neural culture electrophysiological activity, related to Figure 5.

Multiple measures of midbrain neural culture electrophysiological activity (firing rate of active [>5 spikes per minute] neurons, number of active neurons per well) before, during, and after 12-hour exposure to vehicle control or various drugs. Lines represent the means, shaded areas depict ± S.E.M. n = 7-8 wells, from 2 independent neural differentiations. Baseline is hour -1, treatment is hour 12, recovery is hour 22; each data point is one well. Statistical significance determined using a ratio paired t-test.

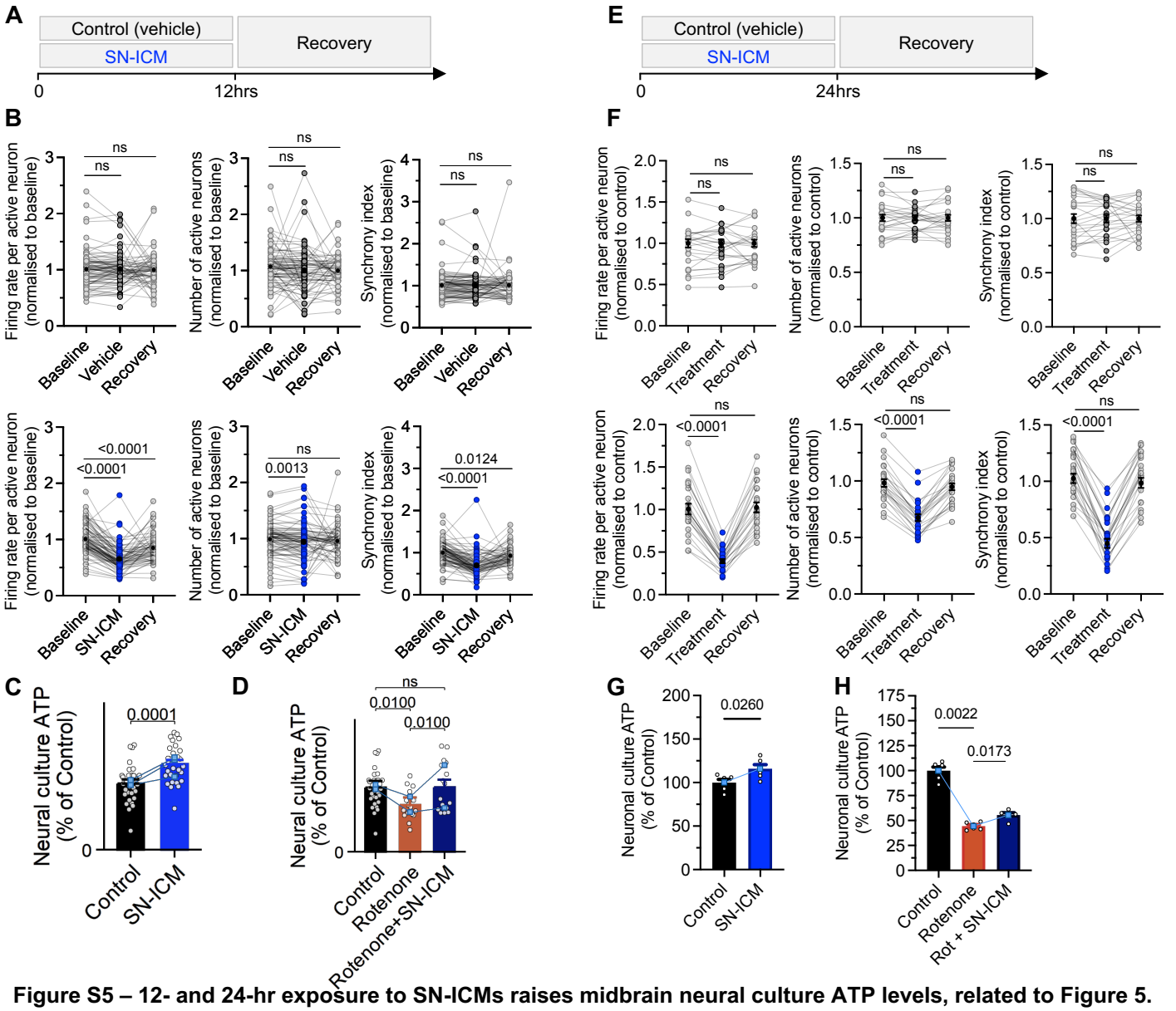

Figure S5 – 12- and 24-hr exposure to SN-ICMs raises midbrain neural culture ATP levels, related to Figure 5. **(A)** Schematic depicting the 12hr treatment and recovery timeline for exposure to the SN-ICM cocktail (SN-ICMs; 10 μM Fisetin, 10 μM ML297, 100 nM α-conotoxin PIA, 100 nM α-conotoxin MII). **(B)** Histograms of the change in firing rate of active neurons (Hz), number of active (>5 spikes per minute) neurons per well, and synchrony index per well before, during and after vehicle (top) or SN-ICM (bottom) exposure. **(C)** ATP levels measured from the lysates of cultures exposed to vehicle control or to SN-ICMs for 12 hours (n= 30-31 technical replicates from 2 independent neural differentiations). Bars represent mean + SEM. Data were normalised to the mean of the control. **(D)** ATP levels measured from the lysates of cultures exposed to vehicle, the concentration of rotenone for a given neural induction corresponding to a 50% reduction (IC_{50}) of culture ATP levels, or co-exposure with rotenone and SN-ICMs for 12 hours. Bars represent the mean + SEM (n = 16-31 wells, from 2-3 independent neural differentiations). Data were normalised to the mean of the vehicle control. Control data is the same as in (C). **(E)** Schematic depicting a 24-hour treatment and recovery timeline for SN-ICM cocktail exposure. **(F)** Histograms of the changes in the same parameters as (B), but with 24hr treatment. **(G)** ATP levels measured from the lysates of cultures exposed to vehicle control or to SN-ICMs for 24 hours (n= 24 technical replicates from 1 independent neural differentiation). Bars represent mean + SEM. Data were normalised to the mean of the control. **(H)** ATP levels measured from the lysates of cultures exposed to vehicle, the concentration of rotenone that gave a 50% reduction (IC_{50}) of culture ATP levels, or co-exposure with rotenone and SN-ICMs for 24 hours. Bars represent the mean + SEM (n = 5-6 wells, from 1 independent neural differentiations). Data were normalised to the mean of the vehicle control. Control data is the same as in (G). **(C,D,G,H)** White circles represent individual well replicates, blue squares with blue paired lines represent averages per neural differentiation. **(C,D)** Statistical significance was determined using linear mixed-effects models, as described in the Methods.. **(G,H)** Statistical significance determined by Mann-Whitney U-test on individual well replicates. **(B,F)** Statistical significance determined using a ratio paired t-test.

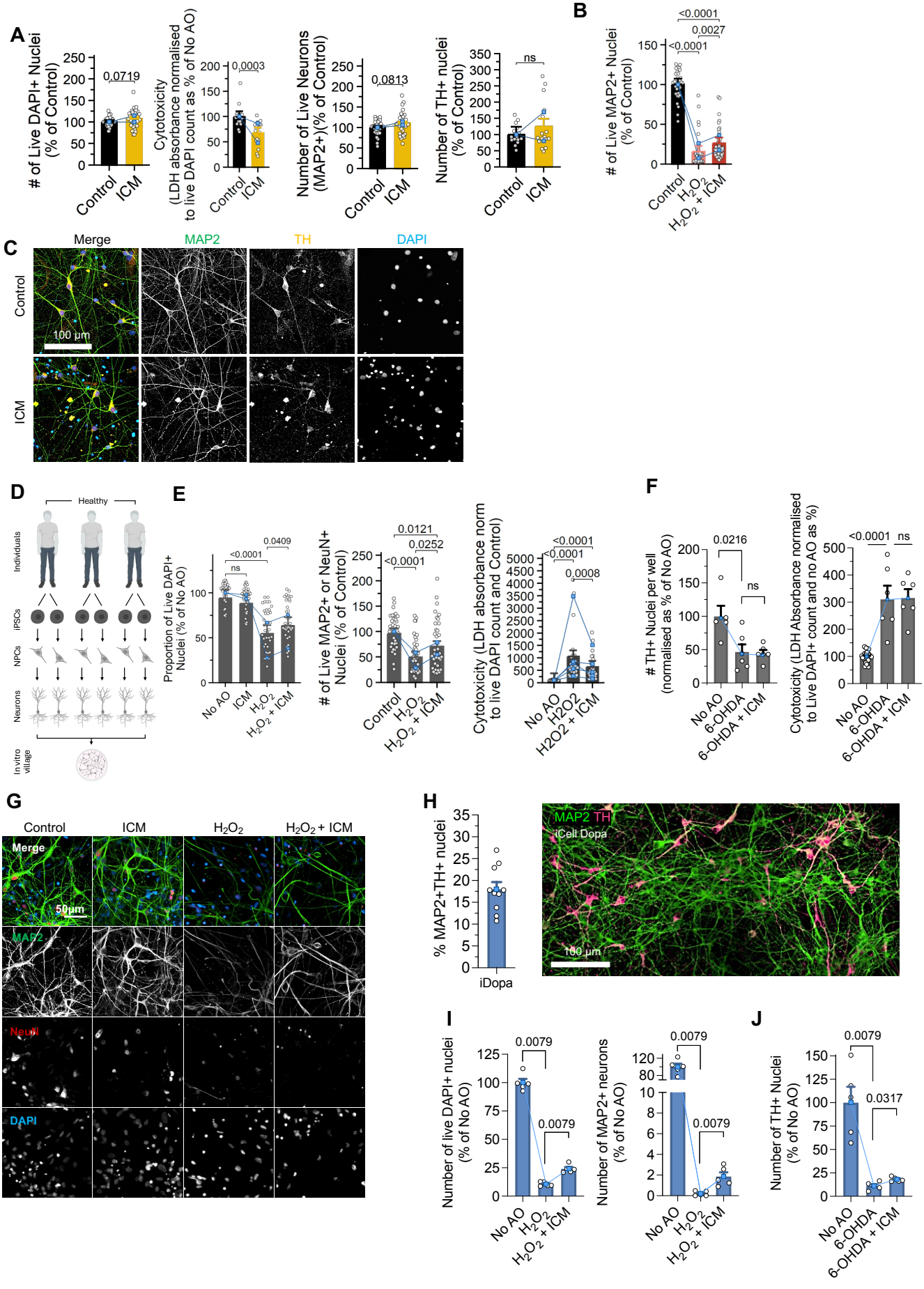
A
# of Live DAPI+ Nuclei (% of Control)
0.0719
Control
ICM
Cytotoxicity (LDH absorbance normalised to live DAPI count as % of No AO)
0.0003
Number of Live Neurons (MAP2+)(% of Control)
0.0813
Number of TH+ nuclei (% of Control)
ns
B
# of Live MAP2+ Nuclei (% of Control)
<0.0001
0.0027
<0.0001
Control
H2O2
H2O2 + ICM
C
Merge
MAP2
TH
DAPI
Control
ICM
100 µm
D
Healthy
Individuals
iPSCs
NPCs
Neurons
In vitro village
E
Proportion of Live DAPI+ Nuclei (% of No AO)
ns
<0.0001
0.0409
No AO
ICM
H2O2
H2O2 + ICM
# of Live MAP2+ or NeuN+ Nuclei (% of Control)
0.0121
0.0252
<0.0001
Control
H2O2
H2O2 + ICM
Cytotoxicity (LDH absorbance norm to live DAPI count and Control)
<0.0001
<0.0001
0.0008
No AO
H2O2
H2O2 + ICM
F
TH+ Nuclei per well (normalised as % of No AO)
0.0216
ns
No AO
6-OHDA
6-OHDA + ICM
Cytotoxicity (LDH Absorbance normalised to Live DAPI+ count and no AO as %)
<0.0001
ns
No AO
6-OHDA
6-OHDA + ICM
G
Control
ICM
H2O2
H2O2 + ICM
Merge
50µm
MAP2
NeuN
DAPI
H
% MAP2+TH+ nuclei
iDopa
MAP2 TH
iCell Dopa
100 µm
I
Number of live DAPI+ nuclei (% of No AO)
0.0079
0.0079
No AO
H2O2
H2O2 + ICM
Number of MAP2+ neurons (% of No AO)
0.0079
0.0079
No AO
H2O2
H2O2 + ICM
J
Number of TH+ Nuclei (% of No AO)
0.0079
0.0317
No AO
6-OHDA
6-OHDA + ICM

Figure S6 – Neuroprotective effects of acute ICM exposure against oxidative stress reproduced in commercial iCell Dopa neural cultures, related to Figure 6.

(A) Neurodegeneration measured as number of live DAPI+ nuclei, lactate dehydrogenase (LDH) absorbance, number of live MAP2+ nuclei per well, and number of live TH+ nuclei of cultures exposed to ICM cocktail for 24 hours. Bars represent means normalised to the control + S.E.M (n= 15-32 wells from 2 independent experiments). **(B)** Number of live MAP2+ cells when exposed to either H_2O_2 or H_2O_2 + ICM for 24 hours. Bars represent means normalised to the control + S.E.M (n= 32-48 wells from 2 independent experiments). **(C)** Representative confocal images of neural cultures exposed to ICMs for 24 hours. Immunocytochemistry labelled neurons and dendrites (MAP2, green), dopaminergic neurons (TH, orange), DAPI labelled nuclei (blue). **(D)** Schematic of in vitro village model. Six iPSC clones from three healthy individuals underwent a neural induction to midbrain dopaminergic neurons and then were pooled. Villages were matured in BrainPhys + supplements for >60 days before experiments. **(E)** Neurodegeneration measured as proportion of live DAPI+ nuclei per well, number of neurons (MAP2+ or NeuN+ nuclei), and lactate dehydrogenase (LDH) absorbance of healthy iPSC-derived neural cultures exposed for 24 hours to ICM, H_2O_2, or both. Bars represent means + S.E.M (n = number of wells from 2 independent experiments). **(F)** Neurodegeneration measured as number of TH+ nuclei and LDH absorbance following 24-hour exposure to 6-hydroxydopamine (6-OHDA; 140μM) with or without ICMs. n= wells from one neural differentiation. **(G)** Representative confocal images of neuronal cultures exposed to H_2O_2 +/- ICMs for 24 hours. Immunocytochemistry labelled neurons and dendrites (MAP2, green), neuronal nuclei (NeuN, red), and DAPI labelled nuclei (blue). **(H)** Quantification of dopaminergic neurons (MAP2+TH+ cells) in iDopa cultures, and representative confocal image showing MAP2 (green) and TH (pink). **(I)** Neurodegeneration measured as number of live DAPI+ nuclei per well and number of MAP2+ neurons following 24-hour treatment with H_2O_2 or H_2O_2 + ICM. **(J)** Dopaminergic neurodegeneration measured as number of TH+ neurons following 24-hour treatment with 6-OHDA (140μM) or 6-OHDA + ICM. **(A-B, E-F, I-J)** Data normalised as percentage of control (No AO). White circles represent individual well replicates, blue squares with blue paired lines represent averages per neural differentiation. **(A,B,E)** Statistical significance was determined using linear mixed-effects models, as described in the Methods. **(F,I,J)** Statistical significance determined by Mann-Whitney U-test on individual well replicates.

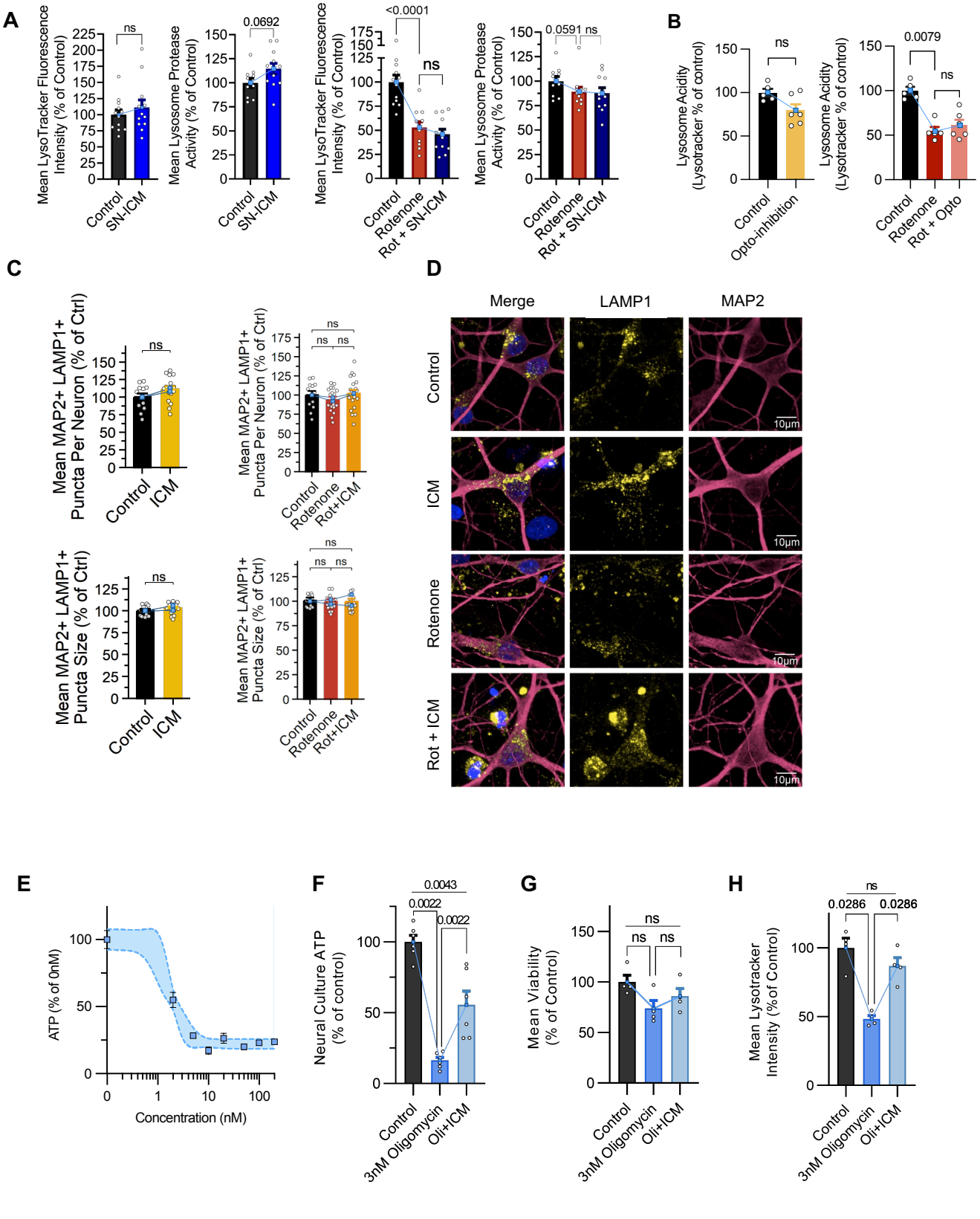
A
Mean LysoTracker Fluorescence Intensity (% of Control)
ns
Control
SN-ICM
Mean Lysosome Protease Activity (% of Control)
0.0692
Control
SN-ICM
Mean LysoTracker Fluorescence Intensity (% of Control)
<0.0001
ns
Control
Rotenone
Rot + SN-ICM
Mean Lysosome Protease Activity (% of Control)
0.0591
ns
Control
Rotenone
Rot + SN-ICM
B
Lysosome Acidity (Lysotracker % of control)
ns
Control
Opto-inhibition
Lysosome Acidity (Lysotracker % of control)
0.0079
ns
Control
Rotenone
Rot + Opto
C
Mean MAP2+ LAMP1+ Puncta Per Neuron (% of Ctrl)
ns
Control
ICM
Mean MAP2+ LAMP1+ Puncta Per Neuron (% of Ctrl)
ns
ns ns
Control
Rotenone
Rot+ICM
Mean MAP2+ LAMP1+ Puncta Size (% of Ctrl)
ns
Control
ICM
Mean MAP2+ LAMP1+ Puncta Size (% of Ctrl)
ns
ns ns
Control
Rotenone
Rot+ICM
D
Merge
LAMP1
MAP2
Control
ICM
Rotenone
Rot + ICM
10µm
E
ATP (% of 0nM)
Concentration (nM)
F
Neural Culture ATP (% of control)
0.0043
0.0022
0.0022
Control
3nM Oligomycin
Oli+ICM
G
Mean Viability (% of Control)
ns
ns ns
Control
3nM Oligomycin
Oli+ICM
H
Mean Lysotracker Intensity (% of Control)
ns
0.0286
0.0286
Control
3nM Oligomycin
Oli+ICM

Figure S7 – Additional effects of generic ICMs, SN-ICMs, and optogenetic inhibition of firing on lysosomes, related to Figure 6.

(A-B) Flow cytometric analysis of Lysotracker Red (lysosome acidity) or Magic Red (protease activity) fluorescence intensity per cell in cultures exposed to various drug treatments. Bars represent the mean + S.E.M. n = number of wells. **(A)** 24-hour exposure to SN-ICM cocktail and rotenone. **(B)** 24-hour exposure to opto-inhibition and rotenone. **(C)** Analysis of MAP2 positive (MAP2+) lysosome quantity and size. Data collected from two experiments from two independent neural inductions: one experiment analysed 19 - 30 20X fields of view per well over 8 - 14 wells per condition; the other analysed 48 40X fields of view per well over 5 - 7 wells per condition. Bars show the well means + SEM. Data were normalised to the means of the vehicle control. Statistical significance was determined using linear mixed-effects models, as described in the Methods. **(D)** Representative confocal images taken at 40X magnification. Immunocytochemistry labelled neurons and dendrites (MAP2, red) and lysosomes (LAMP1, yellow). DAPI labelled nuclei (blue). **(E)** Intracellular ATP levels from cultures exposed to various concentrations of Oligomycin (Oli) for 24 hours. [Inhibitor] vs. normalised response least squares fit was applied to the data in GraphPad Prism; the shaded area indicates the 95% confidence interval. Each data point is the mean of 2 wells from one neural differentiation; error bars indicate ± S.E.M. **(F)** ATP levels measured from the lysates of cultures untreated or exposed to 3nM Oli and ICMs for 24 hours. Bars represent mean + S.E.M. (data points are the number of wells, data from one neural differentiation). Data were normalised to the vehicle control. **(G)** Viability of cultures exposed to Oli and ICMs determined by flow cytometric analysis gating for DAPI- cells. Bars represent the mean + S.E.M. (data points are the number of wells from one neural differentiation). Data were normalised to the mean of the vehicle control. **(H)** Flow cytometric analysis of Lysotracker Red fluorescence intensity per cell in cultures exposed to Oli and ICMs for 24 hours. Bars represent the mean + S.E.M. (data points are the number of wells from one neural differentiation). **(A,B,F,H)** Statistical significance was determined by Mann Whitney U-test. **(A-C,F-H)** White circles represent individual well replicates, blue squares with blue paired lines represent averages per neural differentiation.

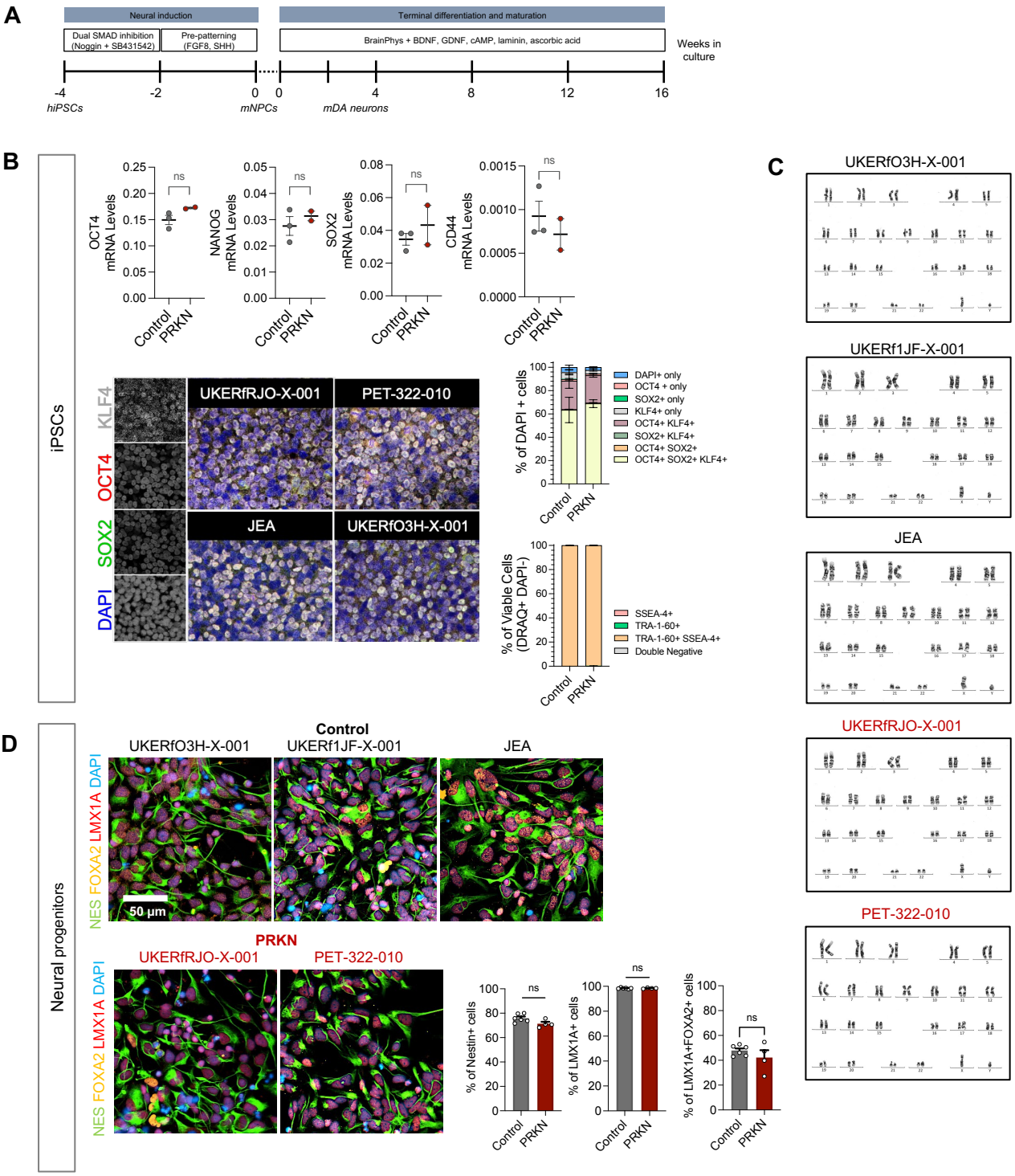

Figure S8 – Validation of differentiation of midbrain dopaminergic neural cultures from patient-derived iPSCs, related to Figure 7.

(A) Timeline of dual SMAD inhibition-based neural induction and BrainPhys-based neural maturation into midbrain dopaminergic neural cultures. **(B)** Expression levels of pluripotency markers in patient-derived iPSCs. OCT4, Nanog, SOX2, and CD44 expression confirmed by qPCR and immunostaining. Proportion of cells in iPSC cultures expressing SSEA-4 and TRA-1-60 was confirmed by flow cytometry. Statistical significance determined by Mann Whitney U Test. **(C)** Karyotyping results per donor. **(D)** Progenitor (Nestin, green) and dopaminergic precursor markers (FOXA2, orange; LMX1A, red) confirmed by immunostaining. Statistical significance determined by Mann Whitney U Test.

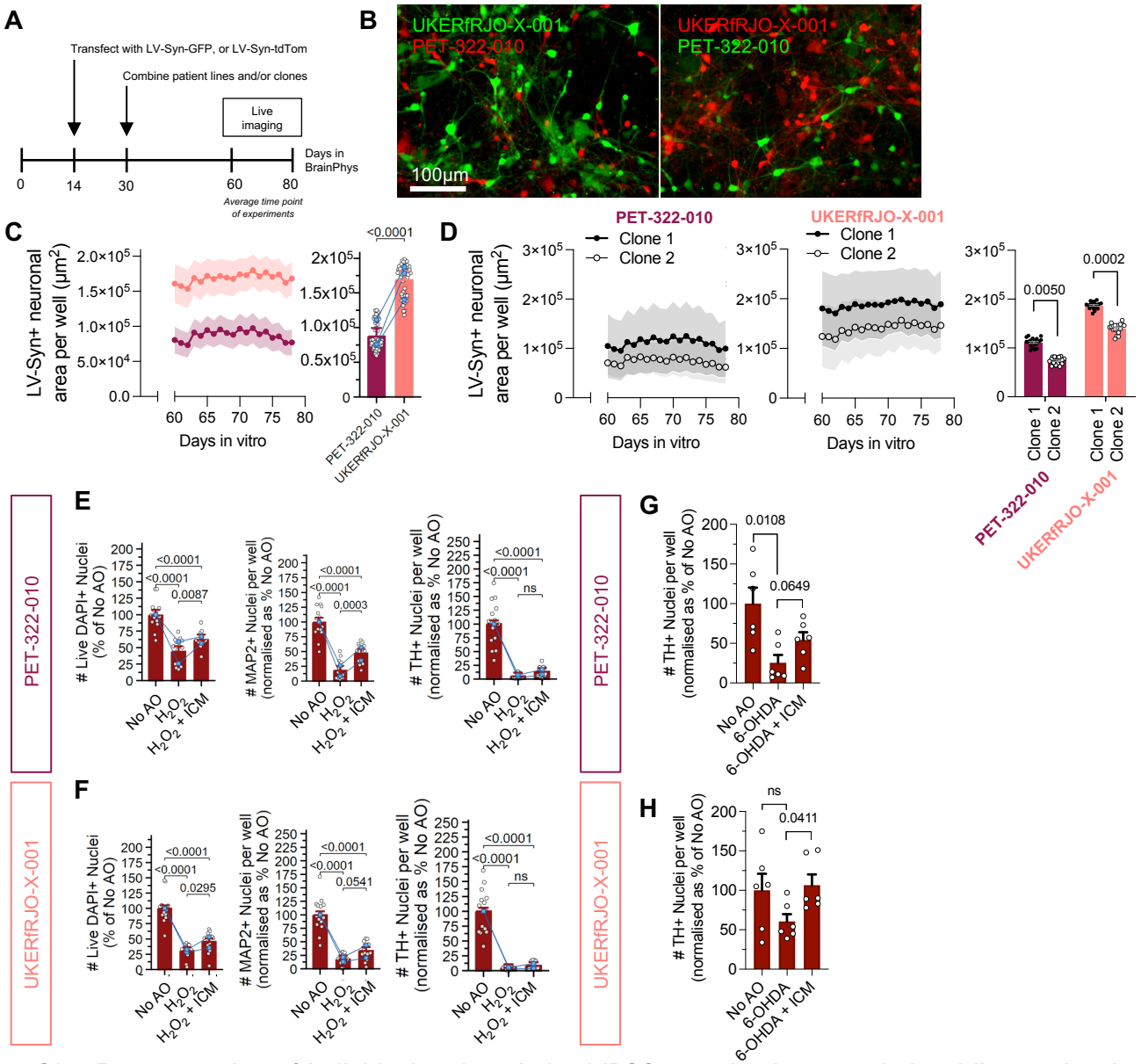

Figure S9 – Representation of individual patient-derived iPSC neural cultures and clonal lines, related to Figure 8.

(A) Experimental paradigm to determine patient contribution to mixed cultures. Clones underwent neural induction and were differentiated into neural cultures. After 14 days in maturation medium (BrainPhys + supplements), cultures were transduced with a Synapsin-driven lentiviral reporter expressing either GFP or tdTomato (pCSC-Synapsin(0.5 kb)-MCS-GFP or pCSC-Synapsin(0.5 kb)-MCS-tdTomato), with each well receiving a single reporter. Two weeks later, cultures were pooled to generate mixed cultures in which neurons from one patient or clone were labelled green and neurons from the other were labelled red. Reciprocal colour assignments were included to control for reporter-specific effects. After 60 days of maturation, mixed cultures were imaged live (Incucyte; Sartorius) for 20 days, and the relative proportions of green- and red-labelled neurons were quantified. **(B)** Representative image of the two patients labelled in green or red, and the reciprocal combination. **(C)** Total area occupied by each patient line, as determined by green- and red-labelled cells, over time (left) and averaged across the imaging period (right). **(D)** Total area occupied by each clone within a patient line, over time (left and middle) and averaged across the imaging period (right). **(C-D)** Bar graphs represent Mean ± SEM. **(E-F)** Neurodegeneration following exposure to H_2O_2 with and without ICMs for each patient line, as measured by DAPI+ count, MAP2+ neuron count, and TH+ dopaminergic neuron count. **(G-H)** Dopaminergic neurodegeneration following exposure to 6-OHDA with and without ICMs for each patient line, as measured by TH+ neuron count. **(C-H)** White circles represent individual well replicates, blue squares with blue paired lines represent averages per neural differentiation. **(C,E,F)** Statistical analysis determined by linear mixed-effect models, as described in methods. **(D,G,H)** Statistical analysis performed using a Mann-Whitney U-test.

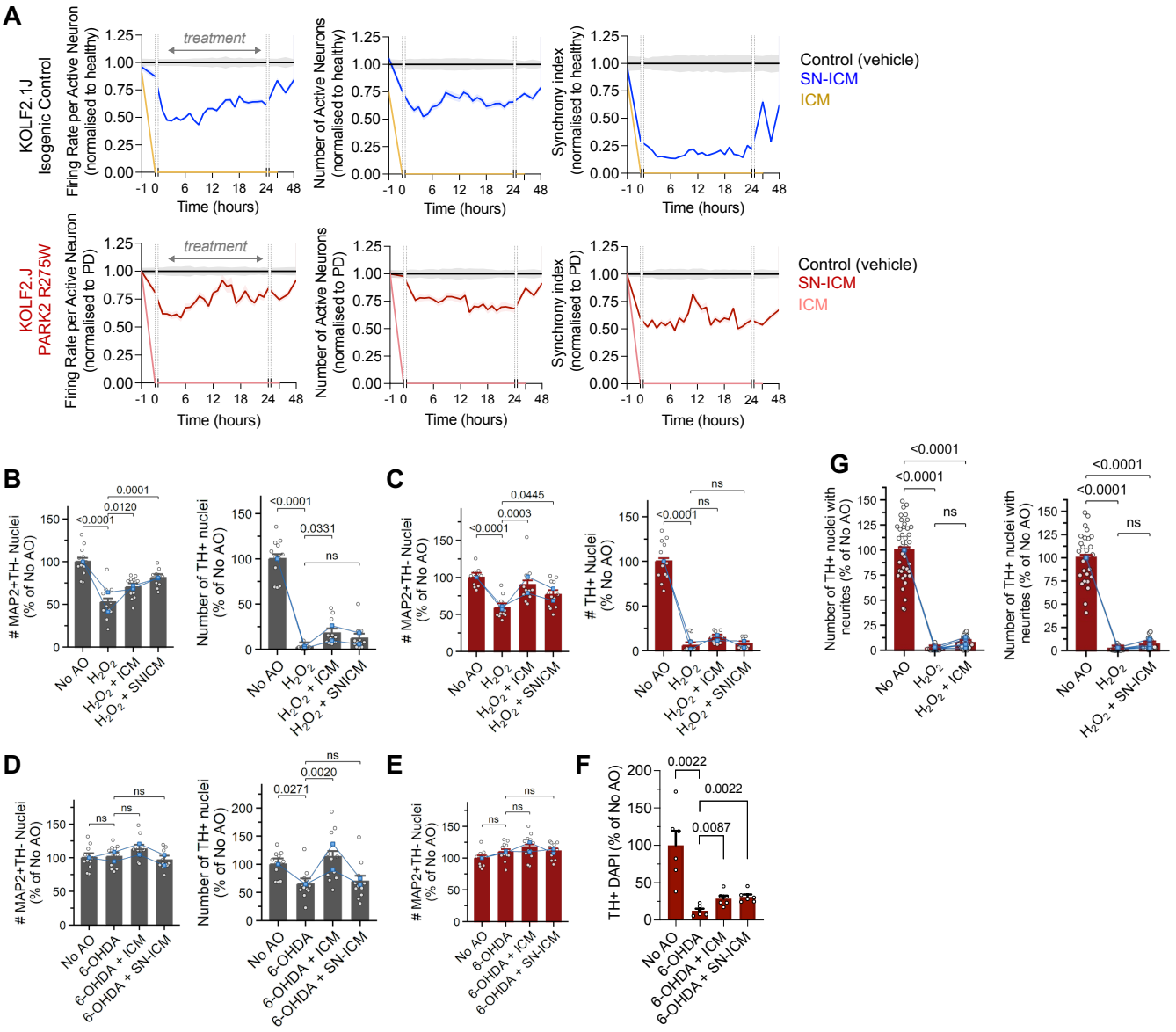

Figure S10 – ICMs and SN-ICMs provide neuroprotection from oxidative stress to both dopaminergic and non-dopaminergic neurons, related to Figure 8.

(A) Firing properties of KOLF2.1J control (top) and KOLF2.1J PARK2 R275W SNV/SNV (bottom) lines over a 24-hour exposure to ICMs or SN-ICMs. Lines represent the mean of 8 wells per condition over two neural differentiations. Shaded regions represent SEM. **(B)** Degeneration as number of TH-negative (left) and TH-positive (right) isogenic control neurons following exposure to H_2O_2 with and without ICMs or SN-ICMs. **(C)** Degeneration as number of TH-negative (left) and TH-positive (right) PARK2-mutant neurons following exposure to H_2O_2 with and without ICMs or SN-ICMs. **(D)** Degeneration as number of TH-negative (left) and TH-positive (right) isogenic control neurons following exposure to 6-OHDA with and without ICMs or SN-ICMs. **(E)** Degeneration as number of TH-negative PARK2-mutant neurons following exposure to 6-OHDA with and without ICMs or SN-ICMs. **(F)** Degeneration as number of TH-positive PARK2-mutant neurons following exposure to 6-OHDA with and without ICMs or SN-ICMs. **(B-E,G)** Data normalised to control (No AO) and presented as a percentage. Bar graphs represent Mean ± SEM. **(B-G)** White circles represent individual well replicates, blue squares with blue paired lines represent averages per neural differentiation. **(B-E, G)** Statistical significance determined using linear missed-effects models, as described in the methods. **(F)** Statistical analysis was performed using a Mann-Whitney U-test.