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## Supplemental Information

# Pluripotent stem cell derived dopaminergic subpopulations model the selective neuron degeneration in Parkinson's disease

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## Supplemental information

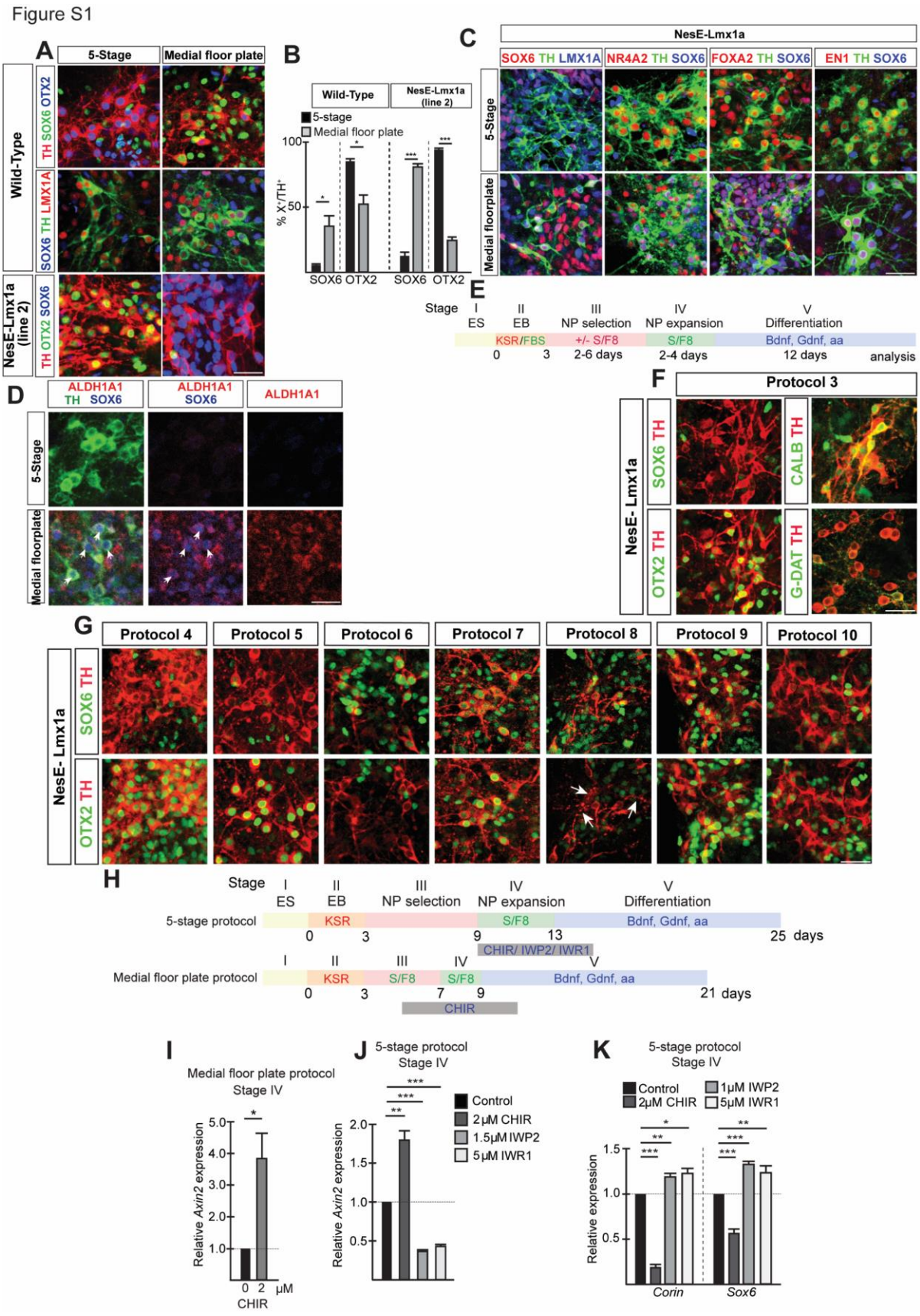

**Figure S1 related to Figures 1 and 2. Different variants of the 5-stage protocol and the effect of Wnt signalling modulation on differentiation outcome. (A)** Expression of DA markers in wild-type and NesE-Lmx1a (line 2) mES cell derived cultures differentiated according the 5-stage and medial floor plate protocols. (**B**) Percentage of SOX6 and OTX2 expressing TH positive neurons in wild-type mES cell and NesE-Lmx1a (line 2) derived cultures from indicated protocols (n = 3 independent experiments); mean values ± SD; unpaired ttest. **(C)** Marker analysis of NesE-Lmx1a ES cells derived TH labelled DA neurons obtained with the 5-stage and medial floor plate protocols. **(D)** Immunohistochemical analysis of ALDH1A1 in $TH^+$ neurons derived from NesE-Lmx1a mES cells obtained with the medial floor plate and 5-stage protocol. Arrows indicate $TH^+/SOX6^+$ neurons expressing ALDH1A1. **(E)** Diagram schematically presenting the different variations of the 5-stage protocol. **(F)** Immunohistochemical analysis of SOX6, OTX2, CALB1 and Glyco-DAT expression in $TH^+$ neurons derived from NesE-Lmx1a mES cells. FBS was used during EB formation in the 5-stage protocol (protocol 3). **(G)** Analysis of SOX6 and OTX2 expression in NesE-Lmx1a mES cell derived $TH^+$ neurons. mES cells were differentiated by different variants on the 5-stage protocol (see **E** and Table S1). Note reduced $TH^+$ cell numbers in cultures differentiated according to protocols 6 and 8. Arrows in protocol 8 indicate deficiently developed SOX6-positive neurons. **(H)** Diagrams showing the timing of CHIR, IWP2 and IWR1 addition in the 5-stage and medial floor-plate protocols. **(I)** Relative gene-expression levels of *Axin2* in NesE-Lmx1a mES cell derived DA progenitors differentiated according to the mFP protocol (Stage IV) upon 4 days exposure to CHIR (Stages III/IV) determined by qPCR (n=3 independent experiments). Mean values ± SD; unpaired ttest. **(J)** Relative gene-expression levels of *Axin2* in NesE-Lmx1a mES cell derived DA progenitors (Stage IV) differentiated according to the 5-stage protocol upon 4 days exposure to CHIR, IWP2 and IWR1 (Stage IV)

determined by qPCR (n=3 independent experiments). Mean values ± SD; one-way ANOVA with Bonferroni correction. **(K)** Relative gene-expression levels of *Corin* and *Sox6* in 5-stage protocol derived DA progenitor cells (Stage IV) exposed to Wnt signalling modulators for 4 days (Stage IV) determined by qPCR (n=3 independent experiments). Mean values ± SD; one-way ANOVA with Bonferroni correction. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar: 50 μM.

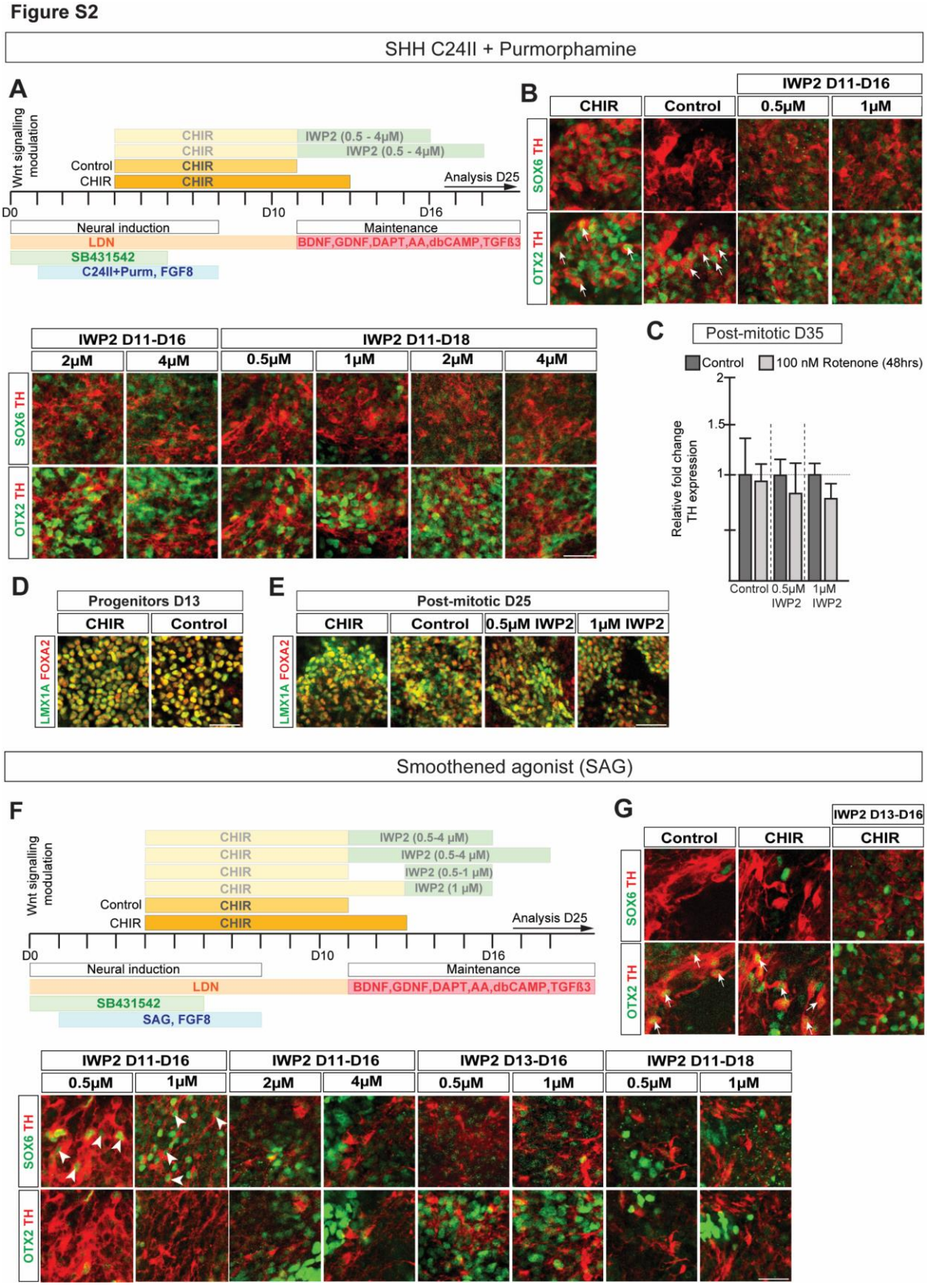


**Figure S2 related to Figure 4. Timing and concentration of IWP2 addition and SHH signalling levels are critical for the efficient induction of SOX6. (A)** Schematic

summary of hES cell differentiation protocol using SHH C24II+purmorphamine during progenitor specification. Conditions: CHIR (CHIR from d4-d13), control (CHIR from d4-d11) and IWP2 (IWP2 from day 11 following CHIR). **(B)** Combinatorial expression analysis of SOX6, OTX2 and TH by immunohistochemistry in SHH C24II+purmorphamine differentiated post-mitotic neurons (d25) following IWP2 treatment. Arrows point to OTX2 expressing TH labelled neurons. **(C)** qPCR analysis of *TH* levels in human ES cell derived DA cultures differentiated as described in **A** after treatment with 100nM rotenone for 48 hrs at d35 (n=3 independent experiments). Mean values ± SD; paired ttest. (**D, E**) Immunohistochemical analysis showing LMX1A and FOXA2 coexpression in progenitors (D13) and post-mitotic DA neurons (D25) after IWP2 (0.5μM and 1μM) exposure. **(F)** Schematic summary of hES cell differentiation protocol using Smoothened agonist (SAG) during progenitor specification. Diagram shows the timing of CHIR and IWP2 addition. Conditions: CHIR (CHIR from d4-d13), control (CHIR from d4-d11), IWP2 (IWP2 from d11 or d13 following CHIR). **(G)** Combinatorial expression analysis using antibodies against SOX6, OTX2 and TH reveals that SOX6 expression increases and OTX2 expression is reduced at D25 following IWP2 treatment when progenitors were differentiated in the presence of SAG. Arrows point to OTX2 expressing TH labelled neurons, while arrowheads indicate SOX6 expressing TH labelled neurons. See **F** for the different conditions that were tested. Note that the optimal IWP2 concentration range is between 0.5 and 1 μM added between d11 and d16. Scale bar: 50 μM.

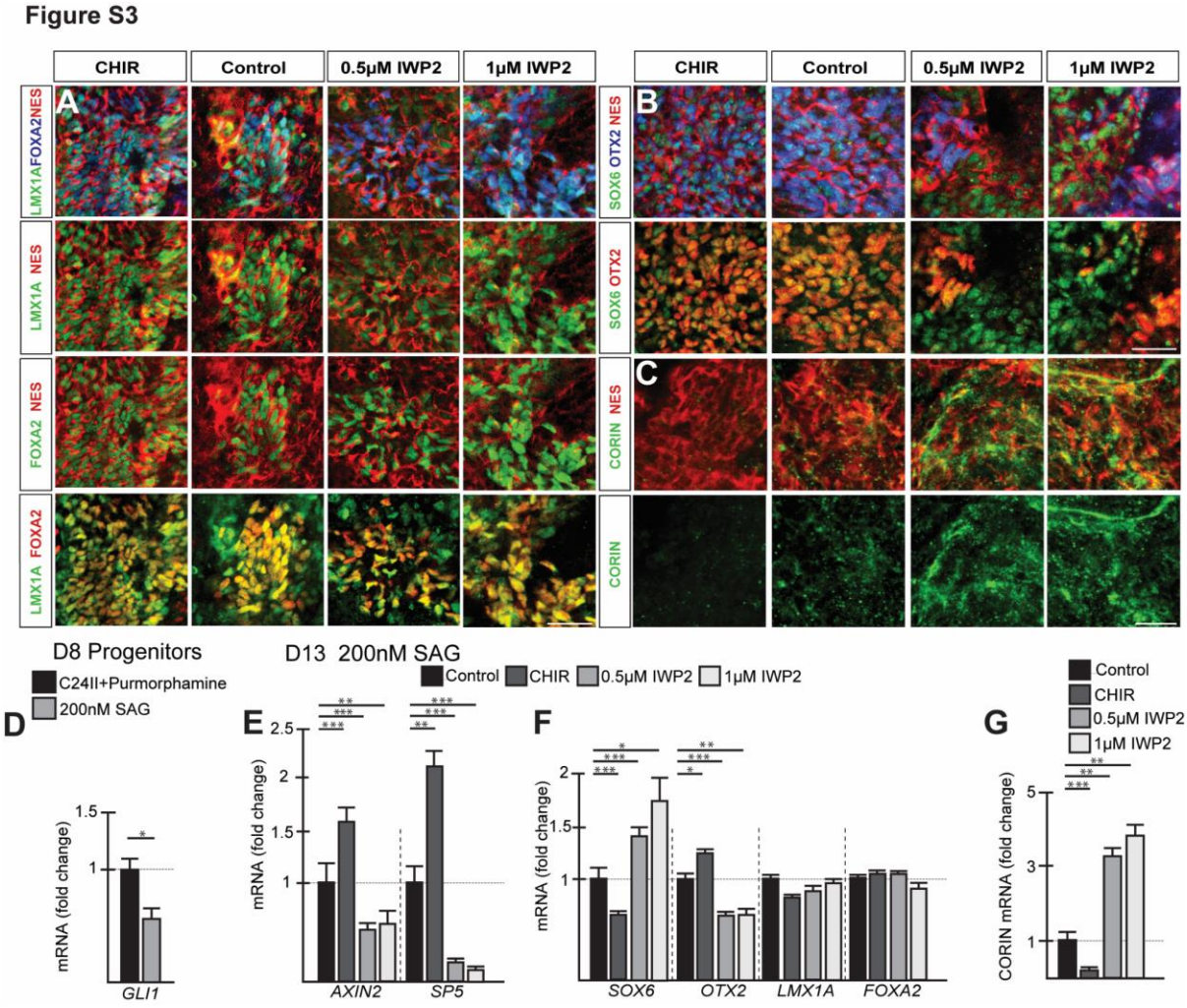


**Figure S3 related to Figure 4. Maintenance of DA progenitor identity in IWP2 treated hES cell derived cultures differentiated in presence of SAG.** (**A-G)** IWP2 was added from d11-d16 as described in **Fig. S4F**. **(A)** Immunohistochemical analysis showing that coexpression of LMX1A and FOXA2 is maintained in NESTIN$^{+}$ progenitors when exposed to IWP2. **(B)** Immunohistochemical analysis of SOX6, OTX2 and NESTIN reveals patches of NESTIN$^{+}$/SOX6$^{+}$/OTX2$^{-}$ progenitors in IWP2 treated cultures. **(C)** Representative images of CORIN and NESTIN immunolabelled progenitors. Note the increase in CORIN expression upon IWP2 treatment. **(D)** Comparison of *GLI1* mRNA levels in D8 progenitors differentiated either in presence of SHH C24II+purmorphamine or SAG. Expression levels were determined by qPCR. Mean values ± SD; unpaired ttest; n=3 independent experiments. **(E)** qPCR analysis of *AXIN2* and *SP5* expression in DA

progenitors (D13) treated with CHIR or IWP2. Gene expression in each condition is normalised to control. Mean values ± SD.; one-way ANOVA with Bonferroni correction; n=3 independent experiments. (**F, G**) qPCR analysis of *SOX6*, *OTX2*, *LMX1A*, *FOXA2* (**F**) and *CORIN* (**G**) expression in hESC derived DA progenitors (D13). Gene expression in each condition is normalised to the control. Mean values ± SD; one-way ANOVA with Bonferroni correction; n=3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar: 50 μM.

Figure S4

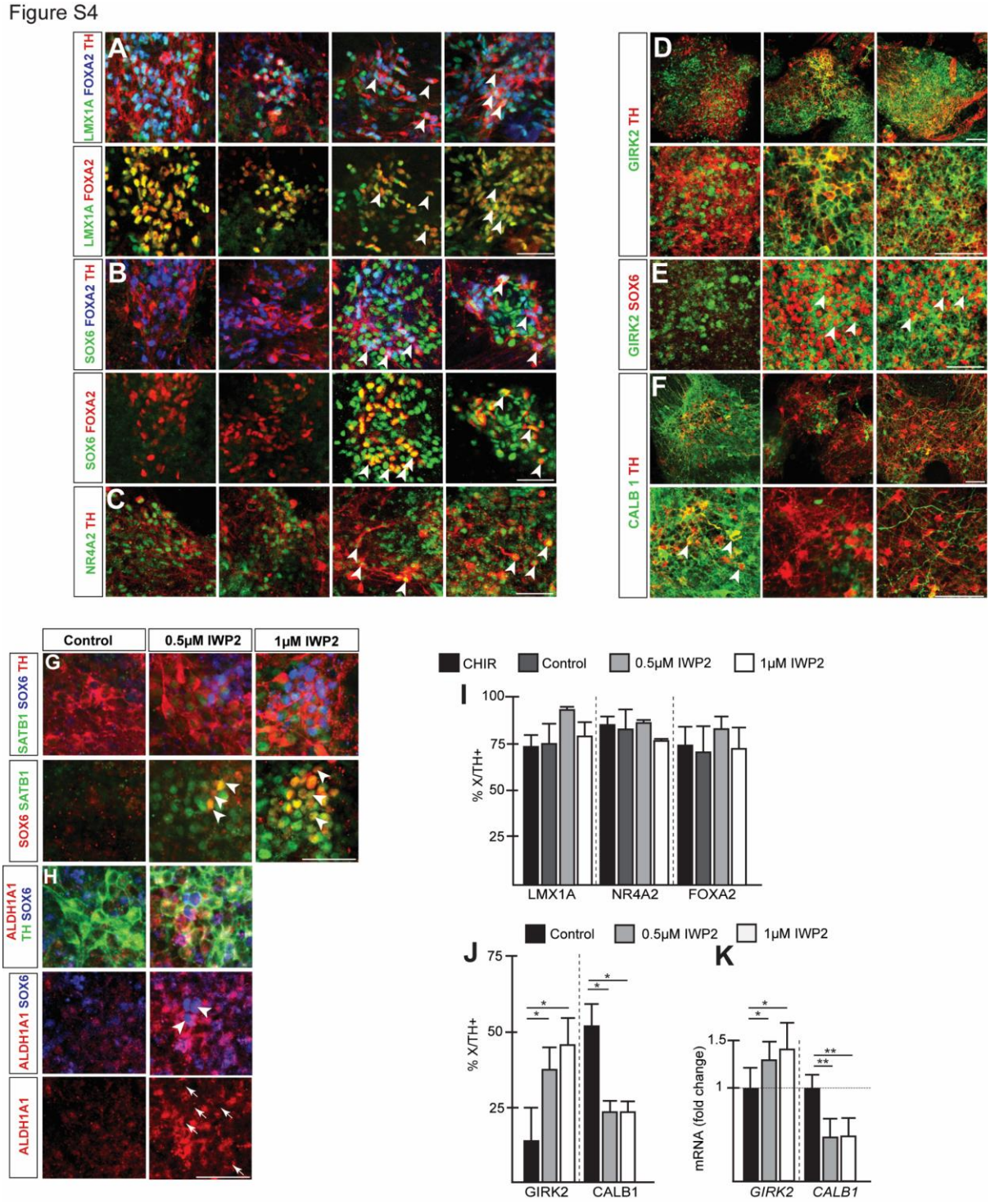


**Figure S4 related to Figure 4. Maintenance of midbrain DA neuronal identity in IWP2 treated hES cell derived cultures differentiated in presence of SAG.** (**A-K)** IWP2 was added from d11-d16 as described in **Fig. S2F**. **(A)** Immunohistochemistry reveals that midbrain DA markers LMX1A and FOXA2 are maintained in TH⁺ hESC derived post-mitotic neurons (d25) following IWP2 exposure. Arrowheads point to

$TH^+/LMX1A^+/FOXA2^+$neurons. **(B)** Immunohistochemistry shows that IWP2 induced $SOX6^+$ post-mitotic $TH^+$ neurons express FOXA2. Arrowheads point to $TH^+/SOX6^+/FOXA2^+$ neurons. **(C)** Midbrain DA marker NR4A2 is expressed in $TH^+/FOXA2^+$ post-mitotic neurons differentiated in presence of IWP2. Arrowheads indicate $TH^+$ neurons coexpressing NR4A2 and FOXA2. **(D, E)** Immunohistochemical analysis shows that GIRK2 expression is induced in $TH^+/SOX6^+$ hESC derived post-mitotic neurons (day 35) after IWP2 exposure. Arrowheads in **D** indicate overlap between GIRK2 and SOX6. **(F)** Immunohistochemical analysis shows that CALB1 expression decreases in hESC derived post-mitotic neurons (day 35) after IWP2 exposure. **(G)** Immunohistochemistry shows that SATB1 is expressed in $SOX6^+TH^+$ neurons (d35) differentiated in presence of IWP2. Arrowheads indicate coexpression of SOX6 and SATB1. **(H)** Immunohistochemical analysis shows coexpression of SOX6 and ALDH1A1 in TH+ neurons following exposure to IWP2 at day 35. Arrowheads point to ALDH1A1+/SOX6+ neurons and arrows point to nuclei of ALDH1A1 labelled neurons. **(I)** Percentage of $TH^+$ neurons expressing LMX1A, NR4A2 or FOXA2 differentiated in control, CHIR and IWP2 treated cultures (D25). Mean values ± SD; n=3 independent experiments. **(J)** Percentage of $TH^+$ neurons at day 35 expressing GIRK2 and CALB1 differentiated in absence (control) or presence of IWP2. Mean values ± SD; one-way ANOVA with Bonferroni correction; n=3 independent experiments. **(K)** qPCR analysis of *GIRK2* and *CALB1* mRNA levels from differentiated DA neurons (D35) after treatment with IWP2 in comparison to control cultures. Mean values ± SD; one-way ANOVA with Bonferroni correction; n=6 independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$. Scale bar: 50 μM.

## Figure S5

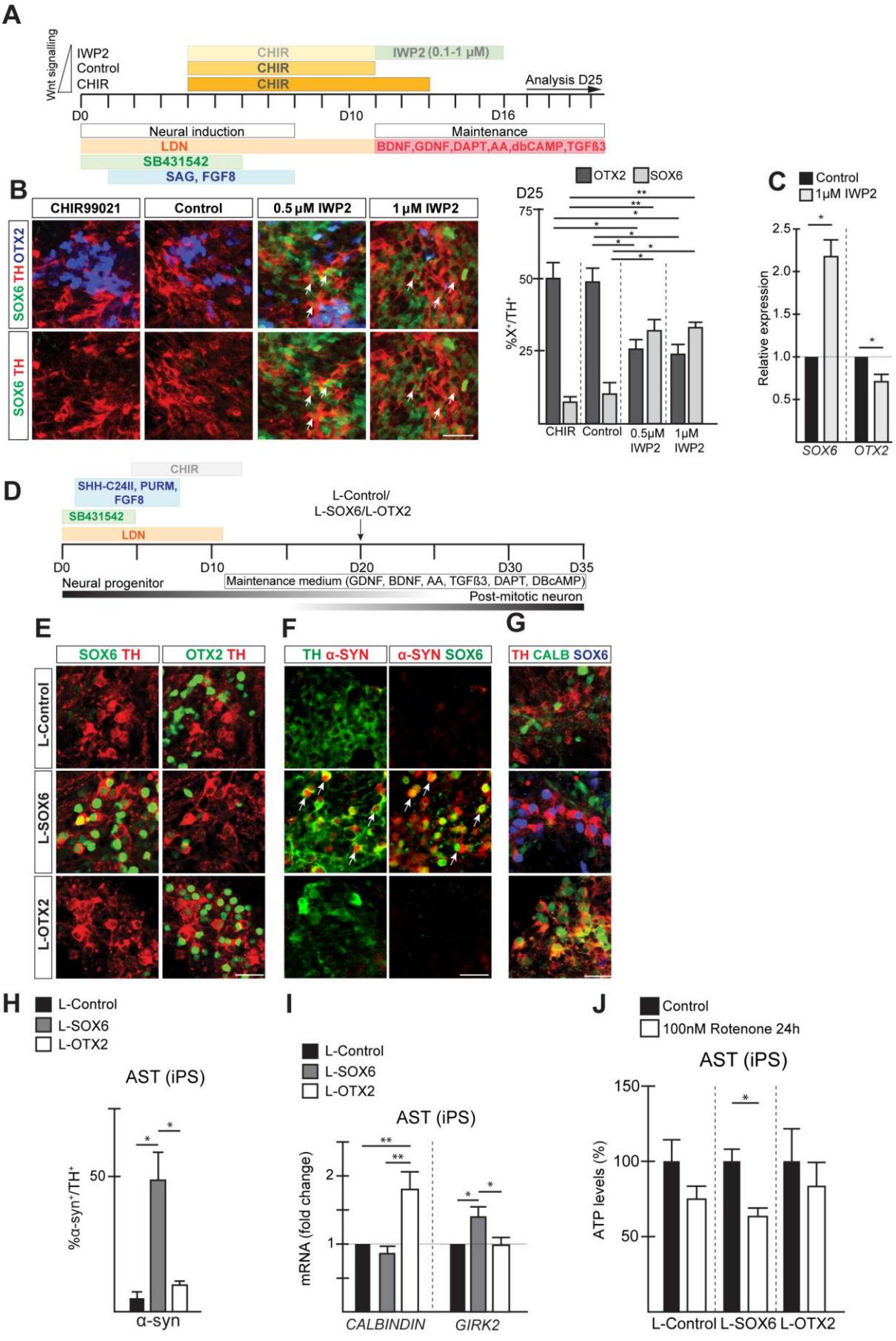
A
Wnt signalling
IWP2
Control
CHIR
CHIR
CHIR
IWP2 (0.1-1 µM)
Analysis D25
D0
D10
D16
Neural induction
Maintenance
LDN
BDNF,GDNF,DAPT,AA,dbCAMP,TGFß3
SB431542
SAG, FGF8
B
CHIR99021
Control
0.5 µM IWP2
1 µM IWP2
SOX6 TH OTX2
SOX6 TH
OTX2 SOX6
D25
%X+/TH+
CHIR
Control
0.5µM IWP2
1µM IWP2
C
Control
1µM IWP2
Relative expression
SOX6
OTX2
D
CHIR
SHH-C24II, PURM, FGF8
SB431542
LDN
L-Control/
L-SOX6/L-OTX2
D0
D10
D20
D30
D35
Neural progenitor
Maintenance medium (GDNF, BDNF, AA, TGFß3, DAPT, DBcAMP)
Post-mitotic neuron
E
SOX6 TH
OTX2 TH
L-Control
L-SOX6
L-OTX2
F
TH α-SYN
α-SYN SOX6
G
TH CALB SOX6
H
L-Control
L-SOX6
L-OTX2
AST (iPS)
%α-syn+/TH+
α-syn
I
L-Control
L-SOX6
L-OTX2
AST (iPS)
mRNA (fold change)
CALBINDIN
GIRK2
J
Control
100nM Rotenone 24h
AST (iPS)
ATP levels (%)
L-Control L-SOX6 L-OTX2

**Figure S5 related to Figure 4 and 5. Reproducibility of methodology in AST iPS cell line. (A)** Schematic summary of iPS cell differentiation protocol using Smoothened agonist (SAG) during progenitor specification. Conditions: CHIR (CHIR from d4-d13), control (CHIR from d4-d11) and IWP2 (IWP2 from day 11 following CHIR). **(B)** Immunohistochemistry showing SOX6 and OTX2 expression in $TH^+$ iPSCs derived post-mitotic neurons (day 25) following exposure to CHIR or IWP2. Arrows indicate $SOX6^+/OTX2^-$ TH labelled neurons in IWP2 treated cultures. Graph displays percentage SOX6 and OTX2 expressing TH labelled neurons under various culture conditions (n=3 independent experiments). mean values ± SD; one-way ANOVA with Bonferroni correction. **(C)** qPCR analysis of *SOX6* and *OTX2* mRNA levels in iPSCs derived DA neurons (D25) treated with 1μM IWP2 (n=4 independent experiments); mean values ± SD; paired ttest. **(D)** Schematic overview of differentiation conditions according to the floor plate protocol. Arrow indicates time point of lentiviral transduction. **(E)** Immunohistochemical analysis of SOX6 and OTX2 in virus transduced iPSCs derived cultures at d35, showing efficient transduction and expression the transcription factors. **(F)** Immunohistochemical analysis of α-SYN and SOX6 in virus transduced iPS cell derived cultures. Arrows point to α-SYN expression in $TH^+$/ $SOX6^+$ neurons. **(G)** Immunohistochemical analysis of CALB1 and SOX6 in virus transduced iPSCs derived cultures at d35, showing increased CALB1 expression in OTX2 but not in SOX6 transduced neurons. **(H)** Percentages of $TH^+$ neurons expressing α-SYN in virus transduced iPSCs derived cultures at d35 (n=3 independent experiments); mean values ± SD; one-way ANOVA with Bonferroni correction. **(I)** qPCR analysis of *CALB* and *GIRK2* mRNA levels in virus transduced iPSCs derived post-mitotic neurons (d35) (n=6 independent experiments); mean values ± SD; one-way ANOVA with Bonferroni correction. **(J)** Graph showing relative ATP levels in virus transduced iPSCs derived post-

mitotic neurons (D35) after treatment with DMSO (Control) or Rotenone (100nM, 24 hrs) (n=3 independent experiments); mean values ± SD; paired ttest. *p<0.05 and **p < 0.01. Scale bar: 50 µM.

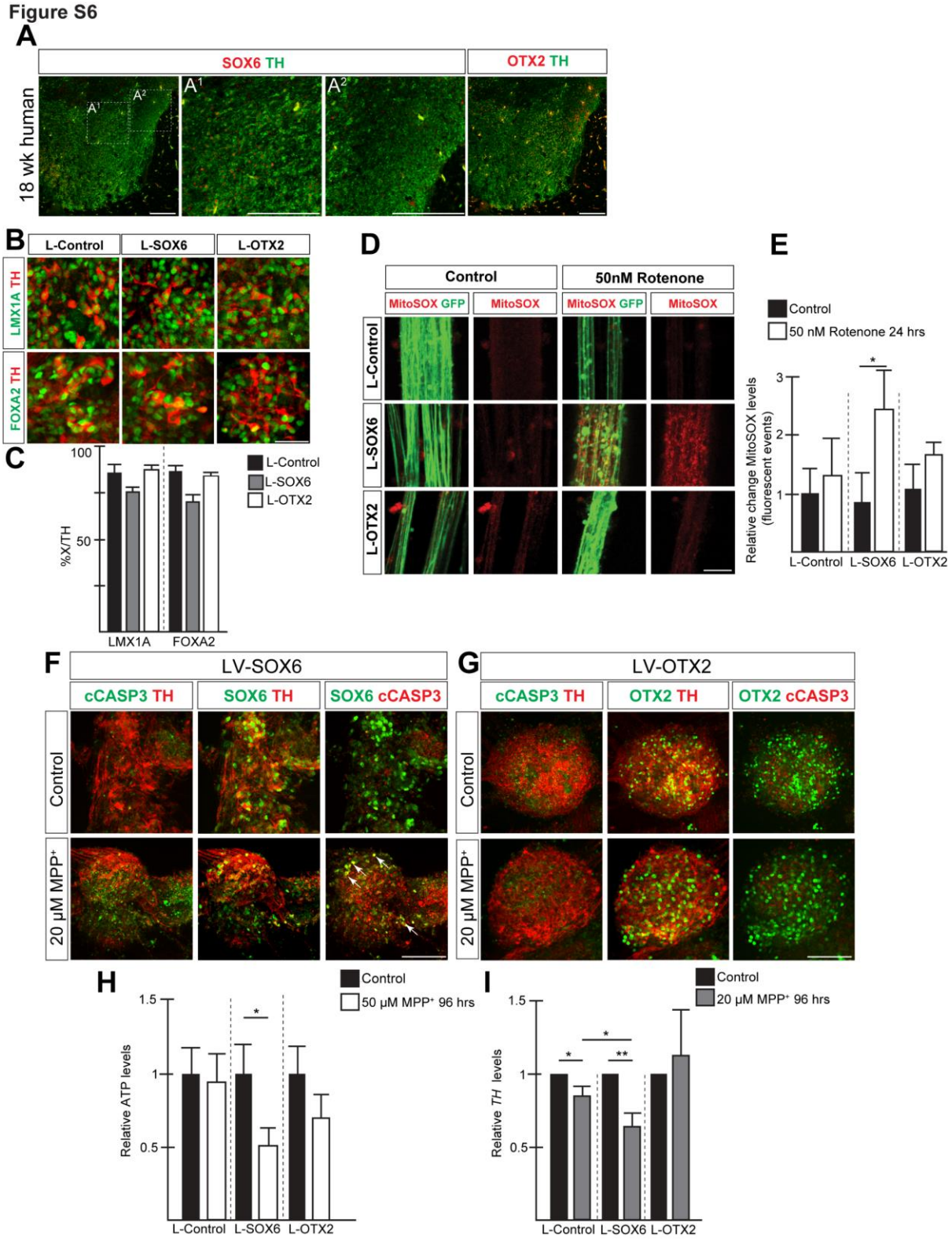

**Figure S6 related to Figure 4, 5 and 6. Increased sensitivity to mitochondrial toxins in L-SOX6 transduced hES cell dopaminergic cultures. (A)** Expression of SOX6 and OTX2 in human VTA dopaminergic neurons. $A^1$ and $A^2$ are higher magnified images of indicated regions. **(B)** LMX1A and FOXA2 expression in TH labelled DA neurons from human ES cell derived cultures (d35) transduced with indicated lentiviruses. **(C)** Percentages of LMX1A and FOXA2 expressing TH neurons after lentiviral transduction. Human ES cell derived cultures of d35. Values are shown as mean ± SD, n=3 independent experiments. **(D, E)** MitoSox analysis of virus transduced cultures treated at D35 with either DMSO or Rotenone for 24 hrs. **(D)** Live imaging of GFP and MitoSox in virus transduced cultures treated with either DMSO or Rotenone for 24 hrs at d35. All cultures were transduced with the lentivirus expressing GFP which enables to visualize the axons. **(E)** Relative fluorescence intensity MitoSox determined by flow cytometry. All values were normalized to untreated cultures transduced with control virus. Values represents the mean ± SD and significance was determined using the paired ttest; n=3 independent experiments. (**F**) Immunofluoresence marker analysis of L-SOX6 transduced cultures treated with $MPP^+$ at d35 for 96 hrs. Arrows point to cCASP3 and SOX6 double labelled cells only observed in $MPP^+$ treated cultures. **(G)** Immunofluoresence marker analysis of L-SOX6 transduced cultures treated with $MPP^+$ at d35 for 96 hrs. **(H)** Graph displays relative ATP levels of control and $MPP^+$ (d35, 96 hrs) treated virus transduced cultures. Values are shown as mean ± SD and significance was determined using the paired ttest; n=4 independent experiments. **(I)** Relative *TH* mRNA levels determined by qPCR. Values are shown as mean ± SD; one-way ANOVA with Bonferroni correction; n=6 independent experiments. *$p<0.05$ and **$p<0.01$. Scale bar: 50 µM (B, D, F, G), 200 µM (A).

**Figure S7**

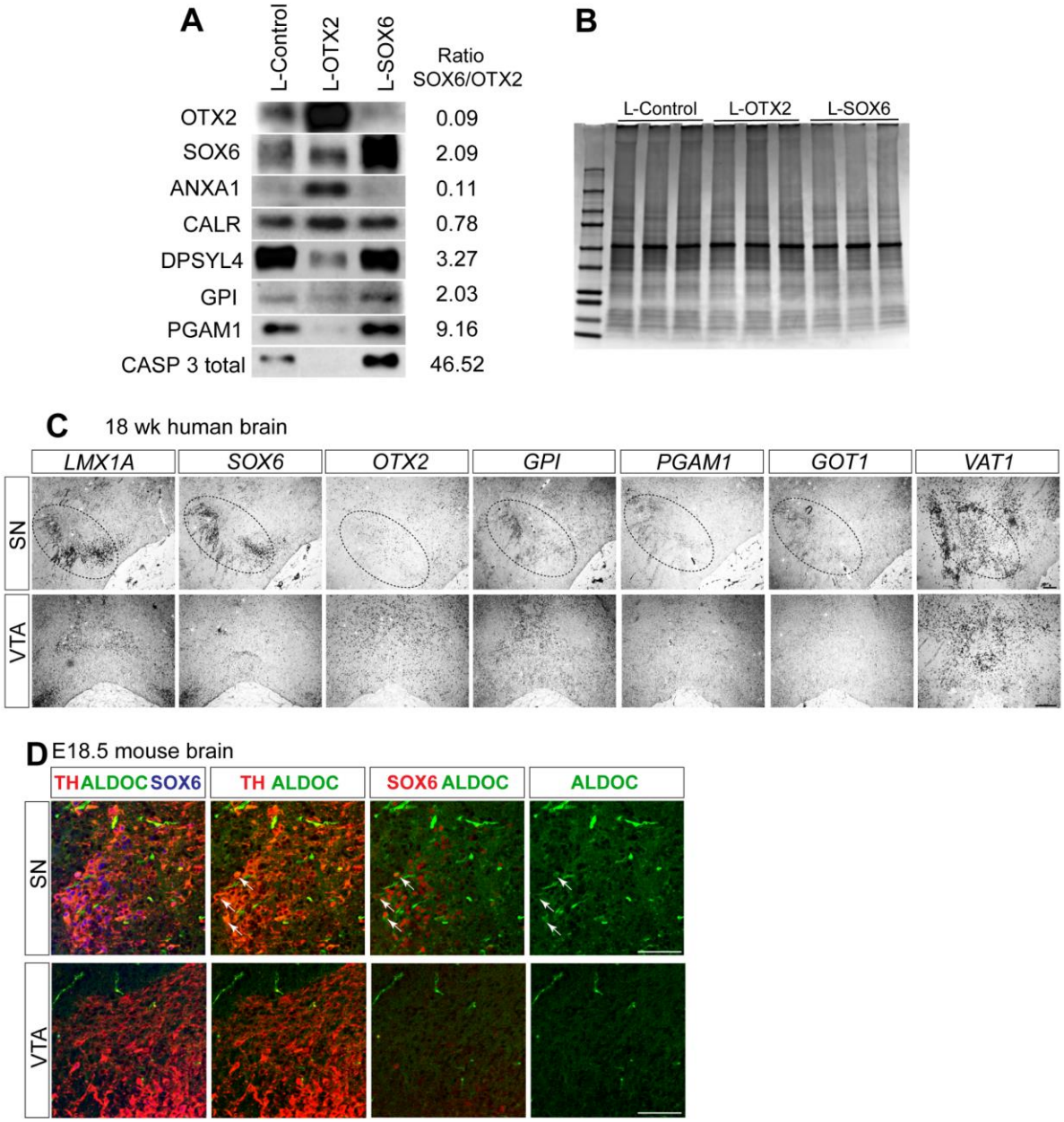


**Figure S7 related to Figure 7. Verification of identified differential expressed proteins. (A)** Western blot analysis show that proteins are expressed as predicted by the Mass-Spec analysis. **(B)** Coomassie Blue analysis that equal proteins quantities were used as an input. **(C)** Analysis of genes involved in metabolism in human foetal midbrain by in situ hybridization. Dashed circle indicate localization of the SN. **(D)** Immunohistochemical analysis of ALDOC and SOX6 expression in SN and VTA of E18.5

mouse brain. Arrows indicate overlap between ALDOC, Sox6 and TH. Scale bar: 100 µM (D), 200 µM (C).

Table S1


| | Stage II | Stage III | | Stage IV | | Analysis stage V | |
|---|---|---|---|---|---|---|---|
| | **EB** | **Conditions** | **N days** | **Conditions** | **N days** | **%Sox6/TH ± SD** | **%Otx2/TH ± SD** |
| **1** | KSR | No growth factors | 6 | SHH,FGF8, | 4 | 8.7 ± 6.7 | 86.9 ± 4.5 |
| **2** | KSR | SHH,FGF8, | 4 | SHH,FGF8, | 2 | 83.1 ± 5.9*** | 11.8 ± 6.80*** |
| **3** | FBS | No growth factors | 6 | SHH,FGF8, | 4 | 8.5 ± 2.9ns | 86.0 ± 12.1ns |
| **4** | FBS | SHH,FGF8, | 4 | SHH,FGF8, | 2 | 8.6 ± 9.7ns | 85.2 ± 12.5ns |
| **5** | KSR | SHH,FGF8, | 6 | SHH,FGF8, | 4 | 12.0 ± 2.0ns | 82.7 ± 3.9ns |
| **6**[1] | KSR | No growth factors | 4 | SHH,FGF8, | 2 | 79.0 ± 11.5 | 7.6 ± 4.6 |
| **7** | KSR | SHH,FGF8, | 4 | SHH,FGF8, | 4 | 18.1 ± 8.6 | 47.3 ± 19.9 |
| **8**[1] | KSR | SHH,FGF8, | 2 | SHH,FGF8, | 2 | 91.0 ± 0.4 | 4.5 ± 0.9 |
| **9** | FBS | No growth factors | 4 | SHH,FGF8, | 2 | 53.0 ± 3.2 | 20.2 ± 0.8 |
| **10** | KSR | No growth factors | 6 | SHH, FGF8 | 2 | 47.3 ± 4.6 | 33.7 ± 6.7 |

**Table S1 related to figure 1 and figure S1.** Overview of the experimental outcomes of the several variants of the 5-stage protocol. Data shows average Sox6 and Otx2 labelled

TH positive neurons. Values are shown as mean ± SD from independent experiments. Protocols 1-5 (n=3) and protocols 6-10 (n=2). Unpaired ttest for protocols 2-5 in comparison to protocol 1. $^{***}p<0.001$; NS=not significant. [1]Note that reduced and deficiently developed $TH^+$ neurons were obtained when differentiated according to protocols 6 and 8 (see figure S1G).

**Supplemental experimental procedures**

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5-stage protocol(Lee et al., 2000) : At the start of the differentiation (stage II) mES cells were separated from MEF cells by plating them on gelatinized tissue culture dishes for 45 minutes. Non-adherent cells were subsequently plated on bacterial dishes for 3 days in medium without LIF in presence of either 10% FBS or KSR. EBs were plated on gelatinized tissue culture dishes (Corning) and allowed to attach. Next day, medium was changed to DMEM/F12 (Gibco) supplemented with insulin (Gibco), apo-transferrin, sodium-selenite and fibronectin (all Sigma) (ITSFn medium; stage III). After 6 days neural precursor cells were splitted with TryplE Express and plated on poly-ornithine and laminin (Sigma) coated 24 well plates containing N3 medium, composed of DMEM/F12, insulin, apo-transferrin, sodium-selenite, progesterone, putrescine and laminin (all Sigma) plus 10 ng/ml bFGF (R&D), 100ng/ml FGF8 (R&D) and 100nM Smoothened agonist (SAG1.3, Calbiochem) (Stage IV). After 4 days neuronal differentiation was initiated by removal of the growth factors and addition of L-ascorbic acid (200 μM, Sigma) (stage V) (See table S1 – Protocol 1 and 3). After 6 days at stage V the medium was further supplemented with BDNF (10 ng/ml) and GDNF (10 ng/ml).

Medial Floorplate protocol: For EB formation the cells were plated in EB medium containing 10% KSR (stage II). After attachment of the EBs medium was changed the next day to N3 medium, plus 10 ng/ml bFGF, 100ng/ml FGF8 and 100nM SAG1.3 (stage III). After 4 days in stage III neural precursor cells were splitted with TryplE Express and plated on poly-ornithine and laminin coated 24 well plates containing N3 medium plus 10 ng/ml bFGF, 100ng/ml FGF8 and 100nM Hedgehog agonist Hh-Ag1.3 (Stage IV). After 2 days growth factors were removed and L-ascorbic acid (200 μM, Sigma) was added

(stage V) (See table S1 – Protocol 2). After 6 days in stage V medium was further supplemented with BDNF (10 ng/ml) and GDNF (10 ng/ml).

Other tested variations of the 5-stage (protocol 4-10): For EB formation either 10% FBS or KSR was added to the medium for 3 days (stage II). After attachment EB medium was changed the next day either to ITSFn, N3 medium or N3 medium plus 10 ng/ml bFGF, 100ng/ml FGF8 and 100nM SAG1.3 (stage III). Cells were kept at stage II between 2-6 days. Then neural precursor cells splitted and plated on poly-ornithine and laminin coated 24 well plates containing N3 medium plus 10 ng/ml bFGF, 100ng/ml FGF8 and 100nM SAG1.3 (Stage IV). After 2-4 days medium was changed to N3 medium containing L-ascorbic acid (200 μM, Sigma) (stage V) and cultured further as described before.

Wnt signalling modulation: 5-stage protocol derived cultures were exposed to 1 μM, 1.5 μM, 2 μM IWP2 or 5 μM IWR1, between days 9-15 (stage IV and V) of differentiation. Medial floorplate derived cultures were exposed to 2 μM CHIR between days 5-11 (stage III, IV and V) of differentiation.

Toxin exposure: For treatment with mitochondrial toxins $MPP^+$ (20 μM) or rotenone (50 nM) were added to the cultures after 12 days in stage V for 48 hrs. Ascorbic acid, BDNF and GDNF were removed from the cultures and DMSO treated cells served as a control. Meclizine (5 μM) was added alone or together with rotenone to the cultures for 48 hrs.

**Maintenance and differentiation of hES and iPS cells**

Maintenance: Human ES cells H9 (WA09, passage 32-48)(Thomson et al., 1998) and iPS cells AST23 (Edi001-A ECACC cat. no. 66540058, passage 25-35)(Devine et al., 2011) were cultured on Geltrex™ (Gibco) coated 6-well plates in Essential 8 medium (Gibco)(Chen et al., 2011) as described by the manufacturer's protocol. Cultures were passaged with 0.5 mM EDTA (Gibco) every 3 days in a 1:6 ratio. Medium was changed

daily. For maintenance of iPS cells 10 µM of Rock inhibitor (Tocris) was added to the medium after the split.

Differentiation of hES and iPS cells according to the floor plate protocol: Human ES and iPS cells were differentiated according to the floor plate protocol(Kriks et al., 2011) with the following modifications: Cells from a confluent well of 6 well plate were transferred to a well of a geltrex coated 24 well plate using EDTA and cultured overnight in Essential 8 medium. The next day neural induction was initiated by replacing the Essential 8 medium with a 1:1 mixture of N2 and B27 containing medium(Shi et al., 2012) supplemented with 100 nM LDN193189 (StemMacs) and 10 µM SB431542 (Tocris). On day 8 progenitors were dissociated with dispase (Life Technologies)(Shi et al., 2012) and cultured in a 24 well plate (1:2 ratio) in the presence of LDN and CHIR. The following concentrations of growth factors were added at a specific time point as previously described(Kriks et al., 2011; see figure 4D and 5B): 100 ng/ml SHH-C24II (R&D), 2 µM Purmorphamine (Calbiochem), 100 ng/ml FGF8 (R&D) and 3 µM CHIR (Stemgent). At day 11 the medium (N2/B27 1:1 mixture) was supplemented with the following survival promoting factors: BDNF (R&D; 25 ng/ml), GDNF (R&D; 25 ng/ml), TGFβ3 (R&D; 1 ng/ml), ascorbic acid (Sigma; 200 µM), DAPT (Sigma; 10 µM), db-cAMP (Sigma; 500 µM) until maturation. CHIR was added from d11 to d13 as previously described (Kriks et al., 2011; see figure 4D and 5B). Between d18 and d20 of differentiation the cultures were split at a 2:3 ratio with Accutase (Millipore) using the manufacturer's protocol and plated on laminin (Sigma), fibronectin (Sigma) and poly-L-ornithine (Sigma) coated plates(Kriks et al., 2011). The medium was changed on a daily basis.

Lentiviral constructs and transduction: cDNAs encoding human *Sox6*, human *Otx2* and GFP (Source Bioscience) were cloned into the lentiviral vector pRRL SIN.cPPT.PGK-GFP.WPRE (Addgene), with a PGK promoter driving transgene expression. The lentiviral

vector without insertion was used as a control. Lentiviral particles were produced in human embryonic kidney cells 293FT cells (Invitrogen) as described before(Panman et al., 2011). Human ES and iPS cell derived cultures were transduced with the virus (MOI=10) the day after the accutase split (between d18 and d20). The next day extra medium was added to the transduced wells and after 48 hrs the virus containing medium was removed and further cultured as described above. Cells were either analysed at d35 or exposed to indicated compounds.

Wnt signalling inhibition: Neural induction was initiated according to the floor plate protocol as described before(Kriks et al., 2011). Progenitors were exposed to either SHH C24II+Purmorphamine or smoothened agonist SAG (Calbiochem; 200nM) and were split at d8 with dispase. Wnt signalling inhibitors were added to specified dopaminergic progenitors at various concentrations (0.5μM, 1μM, 2μM and 4μM) of IWP2 or DKK1 (100 ng/ml). The following time-points of IWP2 treatment were tested (CHIR was removed at d11): d11-d16; d11-d18; d12-d16; d13-d16. For the last time point we removed CHIR either at d11 or at d13 from the culture medium. DKK1 was added from d11 till d16. We referred to the following control conditions: CHIR (CHIR was added until day 13) and control (CHIR was added until day 11). Cultures were kept in maintenance medium till d25 or d35 without splitting.

Toxin exposure: Human ES and iPS cell derived cultures were exposed to various chemical compounds at day 35 for indicated period of time. During toxin exposure BDNF and GDNF were omitted from the culture medium. Lentiviral transduced cultures were exposed to rotenone (50nM and 100nM) for 24 hours and $MPP^+$ (20μM and 50μM) for 96 hours. To alter the glycolytic rate, neurons were exposed to 6PG (10μM) or Meclizine (0.5μM) alone or in combination with rotenone (50nM and 100nM) for 24 hours. Cultures

exposed to Wnt signalling inhibitors were exposed to rotenone (50nM and 100nM) for 48 hours and $MPP^+$ (50μM) for 96 hours at d35.

**Immunohistochemistry**

E11.5 embryos were fixed in 4% PFA for 1h, washed 3 times for 5 min with PBS and put in 30 % sucrose overnight. Tissue was embedded in OCT and sectioned using a cryostat. Sections were air dried for 30 min and frozen at -80 C. Immunohistochemistry in the sections was performed as described(Panman et al., 2014). PS cell derived cultures were fixed with 2% PFA for 20 minutes and immunohistochemistry was performed as described(Panman et al., 2014). Nuclei were visualized by Dapi (Sigma) staining. Stained tissue sections or cells were analysed using a confocal microscope (LSM 510 Meta, Zeiss) or an Axiovert 200M (Zeiss). The antibodies and concentrations are shown in the supplemental information.

**Cell quantification**

Quantification of cells was performed manually from images obtained at 25X magnification. Labelled cells were counted versus the total number of either progenitor, post-mitotic or $DAPI^+$ cells. Cells were counted from at least three different images from three independent experiments and a minimum number of 200 cells per experiment was counted. Measurement were taken from distinct samples. Statistical significance was calculated by two-tailed student's t-test.

**RNA Extraction and quantitative real-time PCR**

Total RNA was extracted using RNeasy micro kit (Qiagen) according to the manufacturers' protocols. Biological replicates were obtained from at least three independent experiments. The RNA quality was assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific) prior to amplification. cDNA was synthesized from total RNA via reverse transcription using the reverse transcriptase SuperScriptIII (Invitrogen) and OligodT as primer (Invitrogen). Real-time qPCR was performed on a QuantStudio 6 Flex system (Thermo Fisher) using Fast SYBR Green Mastermix (Thermo Fisher). Gene expression values were normalized against RPL19 and fold change was calculated using the $2^{-\Delta\Delta CT}$ method(Livak and Schmittgen, 2001). The primer sequences used are shown in the supplementary resource table. Measurement were taken from distinct samples. Significance was determined by the two-tailed student's t-test.

**ATP assay**

By following the manufacturer´s protocol, the CellTiter-Glo Luminescent cell viability assay (Promega) was used to determine the ATP content within lentiviral transduced neurons (control, sox6 and otx2) treated with rotenone and 6PG (see chemical exposure). Measurements were performed in a 96 well plate (Greiner bio-one) with the GloMax 96 microplate luminometer (Promega). Measurement were taken from distinct samples. Significance was determined by the two-tailed student's t-test.

**Mitosox assay**

After rotenone (50nM) treatment for 24 hours, mature lentiviral transduced (GFP, SOX6, OTX2 and control) dopaminergic neurons were incubated, as described in the

manufacturer's protocol, with MitoSOX red reagent (Invitrogen), in order to detect mitochondrial superoxide.

Subsequently, neurons were dissociated with Accutase, transferred to 0.5mL microtubes with HBSS/Ca/Mg buffer (Gibco) and centrifuged at 200g for 10 min. Finally, the pellet was resuspended in PBS (Gibco) and samples were measured by fluorescence activated cell sorting using the BD FACSCanto II Flow Cytometer. Measurement were taken from distinct samples. Significance was determined by the two-tailed student's t-test.

**In-situ hybridisation**

E11.5 wild-type mouse embryos and human embryonic midbrain (CS16 and CS20) were fixed in 4% paraformaldehyde for 2 hours, shaking. After fixation they were incubated in 30% sucrose overnight at 4˚C and subsequently embedded in OCT Compound (VWR). Cryosections (12µM) of mouse and human embryonic brain were made for in situ hybridization. The human in-situ probes AXIN2, LMX1A, SOX6, OTX2, WNT-1 and the mouse in-situ probes Lmx1a, Sox6, Dkk3, Rspo2, Corin were produced by *in vitro* transcription of the linearized plasmid containing the gene of interest. The other listed in-situ probes were made by PCR amplification as previously described(Mong et al., 2014). The in-situ hybridisations were performed following standard procedures(Mong et al., 2014).

**Human tissue analysis**

Human embryos were collected after elective routine abortions with consent given by the pregnant women and approval from the Regional Human Ethics Committee, Stockholm.

Human foetal tissue of Carnegie stage 16, 20- and 18-weeks post conception was obtained from the Institute of Child Health (ICH), University College London. Tissue was fixed for  hrs respectively in 4% PFA at 4°C, incubated in 30% sucrose and mounted in tissue-tek. Cryosections of 12 µM for cs 16 and 20 embryos and 14 µM sections of foetal tissue were made. For immunohistochemical analysis antigen retrieval was performed by incubating the sections for 20 minutes at 98 °C in 10 mM citric acid pH 6.0. Sections were subsequently washed in PBS and blocked with 0.1% triton and 5% donkey serum for 1 hr and incubated with primary antibodies overnight at 4°C, washed with PBS and subsequently incubated with secondary antibodies for one hour at RT.

**Mouse lines**

We used C57/Bl6 animals to obtain wild-type mouse embryonic tissue. The Tcf/Lef::H2B-GFP reporter mouse line(Ferrer-Vaquer et al., 2010) was kept as heterozygous on a C57/Bl6 background. All animal experiments are performed in accordance to the guidelines and legislation as regulated under the Animals Scientific Procedures Act 1986 (ASPA) and were approved by the Animal Welfare and Ethical Review Body (AWERB) of the University of Leicester.

**Western blot  analysis**

Protein concentration was determined using Bradford Reagent (Sigma) and 20 µg of protein were separated by SDS-PAGE on an 8-16% gel and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 hour at RT in 5% low-fat milk in TBS plus 0.5% Tween-20 (TBS-T) and incubated overnight at 4 °C with the primary anti-body diluted in 5% low-fat milk in TBS-T. Membranes were washed in TBS-T and incubated with horseradish peroxidase conjugated secondary antibodies for 1h at RT.

Bands were detected by ECL detecting solution (GE Healthcare) and the intensity of the signal was quantified using the Gel Doc EZ Imager software (Bio-Rad, California, USA).

**Quantitative Mass-Spectrometry analysis**

Sample preparation: For each condition three independent biological replicated were obtained. Protein concentration was determined using Bradford Reagent (Sigma). Each sample was divided over 3 three lanes (40 µg protein/lane) and migrated on a 10% SDS-PAGE gel (50V, 30 minutes). The gel was stained with Coomassie blue and each lane was subsequently cut horizontally in 3 bands and in-gel digested with bovine trypsin (Roche)(Soleilhavoup et al., 2016). Resultant peptides were dried using the SpeedVac.

Nanoflow liquid chromatography tandem mass-spectrometry (NanoLC-MS/MS): All experiments were performed on a dual linear ion trap Fourier transform mass spectrometer (FT-MS) LTQ Orbitrap Velos (Thermo Fisher Scientific) coupled to an Ultimate® 3000 RSLC Ultra High-Pressure Liquid Chromatographer (Thermo Fisher Scientific). Five microliters of each peptide extract was loaded on trap column for desalting and separated using nano-column as previously described(Pini et al., 2016; Soleilhavoup et al., 2016).

Protein identification and validation: Raw data files were processed using Proteome Discoverer software (version 2.1; Thermo Fischer Scientific). Precursor mass range of 350 –5000 Da and signal to noise ratio of 1.5 were the criteria used for generation of peak lists. MS/MS ion searches were performed using Mascot search engine version 2.6 (Matrix Science) against the NCBI nr database (mammalia taxonomy, 2017). Used parameters for database searches were as previously described(Pini et al., 2016; Soleilhavoup et al., 2016). Mascot results from the target and decoy databases searches

were subjected to Scaffold software (version 4.8.9, Proteome Software) using the protein cluster analysis option (assemble proteins into clusters based on shared peptide evidence). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability as specified by the Protein Prophet algorithm and if they contained at least two identified peptides. The abundance of identified proteins within each sample was estimated by calculating the Exponentially Modified Protein Abundance Index (EMPAI).The False Discovery Rate (FDR) was < 0.01 %.

Label-free protein quantification: For comparative analyses, we employed Scaffold Q+ software (version 4.8.4, Proteome Software) to apply two independent quantitative methods: 1) Spectral Counting (SC) which counts and compares the number of fragment spectra identifying peptides of a given protein. 2) Average Precursor Intensity (API), which measures and compares the mass spectrometric signal intensity of peptide precursor ions belonging to a particular protein. Quantification was performed using the "Weighed Spectra" method. Thus, numbers of Normalized Weighed Spectra (NWS) were tabulated using experiment wide protein clustering. The reproducibility linked directly to the nanoLC-MS methodology was evaluated by the coefficients of variance (CV) for the three conditions in triplicate (SOX1-2-3 or OTX 1-2-3 or control 1-2-3) considering 3 technical replicates for each peptide extract analyzed by nanoLC-MS/MS (at total 81 runs) (Supplemental Table S2). Significance between treatments and control was determined using statistical tests within Scaffold Q+ software; Student's t-test for SC and API quantification, where $p < 0.05$ was considered significant. Limits of an average normalized weighted spectra (NWS) of ≥5 and fold change/ratio of ≥2 were included to increase validity of any comparisons made.

Pathway analysis: For pathway analysis L-SOX6 and L-OTX2 transduced neuronal cultures were compared and differentially expressed proteins (t-test $p<0.05$; $fc>2$) of both cell types were analysed for enrichment of their corresponding genes associated with pathways using ToppGene Server(Chen et al., 2009b).

**References:**


Chen, J., Bardes, E.E., Aronow, B.J., and Jegga, A.G. (2009b). ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res *37*, W305-311.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E*., et al.* (2011). Chemically defined conditions for human iPSC derivation and culture. Nat Methods *8*, 424-429.

Ferrer-Vaquer, A., Piliszek, A., Tian, G., Aho, R.J., Dufort, D., and Hadjantonakis, A.K. (2010). A sensitive and bright single-cell resolution live imaging reporter of Wnt/ss-catenin signaling in the mouse. BMC Dev Biol *10*, 121.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Pini, T., Leahy, T., Soleilhavoup, C., Tsikis, G., Labas, V., Combes-Soia, L., Harichaux, G., Rickard, J.P., Druart, X., and de Graaf, S.P. (2016). Proteomic Investigation of Ram Spermatozoa and the Proteins Conferred by Seminal Plasma. J Proteome Res *15*, 3700-3711.

Shi, Y., Kirwan, P., and Livesey, F.J. (2012). Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. Nat Protoc *7*, 1836-1846.

**Key resources table**

| Gene | Mouse qPCR primer sequences | |
|---|---|---|
| *Axin2* | GCTCACCCATTTACCCAGGAC | TTCCTGTCCCTCTGCTGACTG |
| *Corin* | CTGCTCATTTTGCCAAGACA | ACAGCCCCATTCATCAGAAC |
| *Lmx1a* | TGCCTGGAGATCACATGCAC | CATATGGGAGCCCAGGTCAC |
| *Otx2* | AGAATCCAGGGTGCAGGTATG | TGGAGAGCTCTTCTTCTTGGC |
| *RPL19* | GGTGACCTGGATGAGAAGGA | TTCAGCTTGTGGATGTGCTC |
| *Sox6* | GTGTTTGCCTCTTGATGTGCC | TGGATGTAGTGAGAGGCGGTC |
| *TH* | GCTGGAGGATGTGTCTCACTTCT | CAGAAAATCACGGGCAGACAGTA |

| Gene | Human qPCR primer sequences | |
|---|---|---|
| *AXIN2* | ACAACAGCATTGTCTCCAAGCAGC | GCGCCTGGTCAAACATGATGGAAT |
| *CALB1* | CACAGCCTCACAGTTTTTCG | CCTTTCCTTCCAGGTAACCA |
| *CORIN* | CATATCTCCATCGCCTCAGTTG | GGCAGGAGTCCATGACTGT |
| *FOXA2* | TGGGAGCGGTGAAGATGGAAGGGCAC | TCATGCCAGCGCCCACGTACGACGAC |
| *GIRK2 (KCNJ6)* | AGGAGATCATGATTGAGTGAAGC | GGCCATTGTTGCAGTTTCTT |
| *GLI1* | AAGCGTGAGCCTGAATCTGT | GATGTGCTCGCTGTTGATGT |
| *LMX1a* | CAGCCTCAGACTCAGGTAAAAGTG | TGAATGCTCGCCTCTGTTGA |
| *OTX2* | GGGTATGGACTTGCTGCAC | CCGAGTGAACGTCGTCCT |
| *RPL19* | TGAGACCAATGAAATCGCCAATGC | ATGGACCGTCACAGGCTTGC |
| *SOX6* | GCTTCTGGACTCAGCCCTTTA | (Rev) GGCCCTTTAGCCTTTGGTTA |
| *SP5* | TCGGACATAGGGACCCAGTT | (Rev) CTGACGGTGGGAACGGTTTA |
| *TH* | TCATCACCTGGTCACCAAGTT | (Rev) GGTCGCCGTGCCTGTACT |

**Primary antibodies**

| Antibodies | Species | Company + Identifier | Dilution |
|---|---|---|---|
| α-SYN | Mouse | BD Biosciences (610786) AB_398107 | 1:500 |
| Aldh1a1 | Rabbit | Sigma (HPA050139) AB_2681031 | 1:500 |
| Aldoc1 | Mouse | Santa Cruz (sc-271593) | 1:1000 |
| Annexin A1 | Mouse | Santa Cruz (sc-12740) AB_2057007 | WB 1:1000 |
| CALBINDIN | Rabbit | Swant (CB38) AB_2721225 | 1:500 |
| Calreticulin | Rabbit | Abcam (ab2907) AB_303402 | WB 1:1000 |
| cCASP-3 | Rabbit | Cell Signaling (9579S) AB_10897512 | 1:500 |
| cCASP-3 | Rabbit | Cell Signaling (9664S) AB_2070042 | WB 1:1000 |
| CORIN | Rat | R&D Systems (MAB2209) AB_2082224 | 1:500 |
| Dpsyl4 (CRMP3) | Rabbit | Abcam (ab133287) AB_11154751 | WB 1:1000 |
| ENGRAIL-1 | Mouse | DSHB (4G11) AB_528219 | 1:20 |
| FOXA2 | Goat | R&D Systems(AF2400) AB_2294104 | 1:500 |
| γ-SYN | Rabbit | Abcam (ab55424) AB_2193398 | 1:3000 |
| GFP | Rabbit | Abcam (ab6556) AB_305564 | 1:1000 |
| GIRK2 (KCNJ6) | Goat | Abcam (ab65096) AB_1139732 | 1:500 |
| GPI | Mouse | Santa Cruz (sc-365066) AB_10841426 | WB 1:1000 |
| Glyco-DAT | Rat | Merck Millipore (MAB369) AB_2190413 | 1:500 |
| LMX1a | Rabbit | Merck Millipore (AB10533) AB_10805970 | 1:1000 |
| LMX1a | Guinea Pig | gift from Johan Ericson | 1:10000 |
| NESTIN | Mouse | BD Biosciences (611658) AB_399176 | 1:1000 |
| NURR1 | Rabbit | Santa Cruz (sc-990) AB_2298676 | 1:1000 |
| OTX2 | Goat | R&D Systems (AF1979) AB_2157172 | 1:500 |
| PGAM1 | Rabbit | Cell Signaling (12098S) AB_2736922 | WB 1:1000 |
| SATB1 | Rabbit | Abcam (ab7004) AB_955479 | 1:250 |
| SOX6 | Guinea pig | gift from Jonas Muhr | 1:1500 |
| SOX6 | Mouse | Santa Cruz (sc-393314) N/A | WB 1:1000<br>IH 1:500 |
| SOX6 | Rabbit | Atlas Antibodies (HPA001923) AB_1080065 | 1:1000 |
| TH | Mouse | Merck Millipore (MAB318) AB_2201528 | 1:1000 |
| TH | Rabbit | Pel-Freez (P40101-150) AB_2617184 | 1:500 |
| TH | Sheep | Pel-Freez (P60101-150) | 1:500 |

**Secondary antibodies**

| | | | |
|---|---|---|---|
| Anti-Goat Alexa Fluor 488 | Donkey | Invitrogen (A-11055) | 1:500 |
| Anti-Goat Alexa Fluor 555 | Donkey | Invitrogen (A-21432) | 1:500 |
| Anti-Goat Alexa Fluor 647 | Donkey | Invitrogen (A-21447) | 1:500 |
| Anti-Mouse Alexa Fluor 488 | Donkey | Invitrogen (A-21202) | 1:500 |
| Anti-Mouse Alexa Fluor 555 | Donkey | Invitrogen (A-31570) | 1:500 |
| Anti-Rabbit Alexa Fluor 488 | Donkey | Invitrogen (A-21206) | 1:500 |
| Anti-Rabbit Alexa Fluor 555 | Donkey | Invitrogen (A-31572) | 1:500 |
| Anti-Rat Alexa Fluor 488 | Donkey | Invitrogen (A-21208) | 1:500 |
| Anti-Guinea pig Cy5 | Donkey | Stratech (706-175-148-JIR) | 1:800 |

**Primers designed for in-situ hybridization probe synthesis**

| **Gene** | **Primer sequences** | |
|---|---|---|
| h*CORIN* | ATGGTGACGAGGACTGCAAG | TCACTCACCTAAGCAGCCTGA |
| h *VAT1* | TTGAACCGGTCAGGGATGTG | TTCCCAACTTCTCCCTTCGC |
| m *Wnt1* | ATGCGCCAAGAGTGCAAATG | CGCTATGAACCCTGGGACTG |

**Other reagents**

| Reagents | Supplier | Cat number |
|---|---|---|
| **Non-adherent bacterial dishes** | Thermo Fisher | 101R20 |
| **100mm tissue culture dishes** | Corning | 3296 |
| **24 well plates (mouse)** | Corning | 3337 |
| **Nunc- 24 well plates (human)** | Thermo scientific | 142475 |
| **Nunc- 6 well plates (human)** | Thermo scientific | 140675 |
| **96 well plates (ATP assay)** | Greiner bio-one | 655095 |
| **Accutase** | Millipore | SCR005 |
| **Ascorbic acid** | Sigma | A4403-100MG |
| **ATP assay ( CellTiter-Glo Luminescent cell viability assay)** | Promega | G7570 |
| **B27 supplement** | Life technologies | 17504-044 |
| **BDNF** | R&D | 248-BD-025 |
| **bFGF** | R&D | 233-FB |
| **CHIR99021** | Stemgent | 04-0004-10 |
| **DAPI** | Sigma | D9564 |
| **DAPT** | Sigma | D5942-25MG |
| **Dibutyril cAMP** | Sigma | D0627-250MG |
| **Dispase** | Life technologies | 17105041 |
| **Dimethyl sulfoxide (DMSO)** | Sigma | D8418 |
| **DKK1** | R&D | 5439-DK-010 |
| **DMEM high glucose** | Gibco | 41965-039 |
| **DMEM/F12 Glutamax** | Gibco | 31331-028 |
| **DMEM/F12** | Gibco | 10565-018 |
| **DPBS -Ca -Mg** | Gibco | 14190-094 |
| **Essential 8** | Gibco | A1517001 |
| **Fetal bovine serum (FBS)** | Gibco | 16141-079 |
| **FGF8** | R&D | 423-F8-01M |
| **Fibronectin** | Sigma | F0895 |

| | | |
|---|---|---|
| **Geltrex** | Gibco | A1413302 |
| **GDNF** | R&D | 212-GD-050 |
| **HBSS/Ca/Mg** | Gibco | 14025-050 |
| **Insulin** | Gibco | 12585-014 |
| **IWP2** | Tocris | 3533 |
| **IWR1** | Tocris | 3532 |
| **KnockOut Serum Replacement (KSR)** | Gibco | 10828028 |
| **Laminin** | Sigma | L2020 |
| **LDH cell viability assay** | Promega | G7890 |
| **LDN193189** | StemMacs | 130-103-925 |
| **L-Glutamine** | Gibco | 25030-024 |
| **L(+)-Ascorbic Acid** | Sigma | A7631 |
| **LIF** | Merck Millipore | ESG1107 |
| **Meclizine dihydrochloride** | Tocris | 4245 |
| **β-Mercaptoethanol** | Sigma | M3148 |
| **MitoSOX** | Invitrogen | M36008 |
| **MPP dihydrochloride hydrate** | Sigma | M7068 |
| **N2 supplement** | Gibco | 17502-048 |
| **Neurobasal** | Gibco | 12348-017 |
| **Non-essential amino acids** | Gibco | 11140-050 |
| **Paraformaldehyde** | Sigma | P6148 |
| **Penicillin-Streptomycin** | Thermo Fisher | 15140-122 |
| **Poly-L-ornithine** | Sigma | P4957 |
| **Progesterone** | Sigma | P6149 |
| **Purmorphamine** | Calbiochem | 540220-5 |
| **Putrescine dihydrochloride** | Sigma | P5780 |
| **Rotenone** | Sigma | R8875 |
| **SHH-C24II** | R&D | 1845-SH-500 |
| **SAG1.3** | Calbiochem | 566660 |
| **SB431542** | Tocris | 1614/10 |
| **Sodium selenite** | Sigma | S9133 |
| **TGFβ3** | R&D | 243-B3-010 |

| | | |
|---|---|---|
| **6PG (6-Phosphogluconic acid trisodium salt)** | Sigma | P6888 |
| **TryplE Express** | Gibco | 12604-013 |
| **UltraPure EDTA, pH 8.0** | Gibco | 15575-020 |