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Supplemental Information

Direct Reprogramming of Human Fetal- and Stem Cell-Derived Glial Progenitor Cells into Midbrain Dopaminergic Neurons

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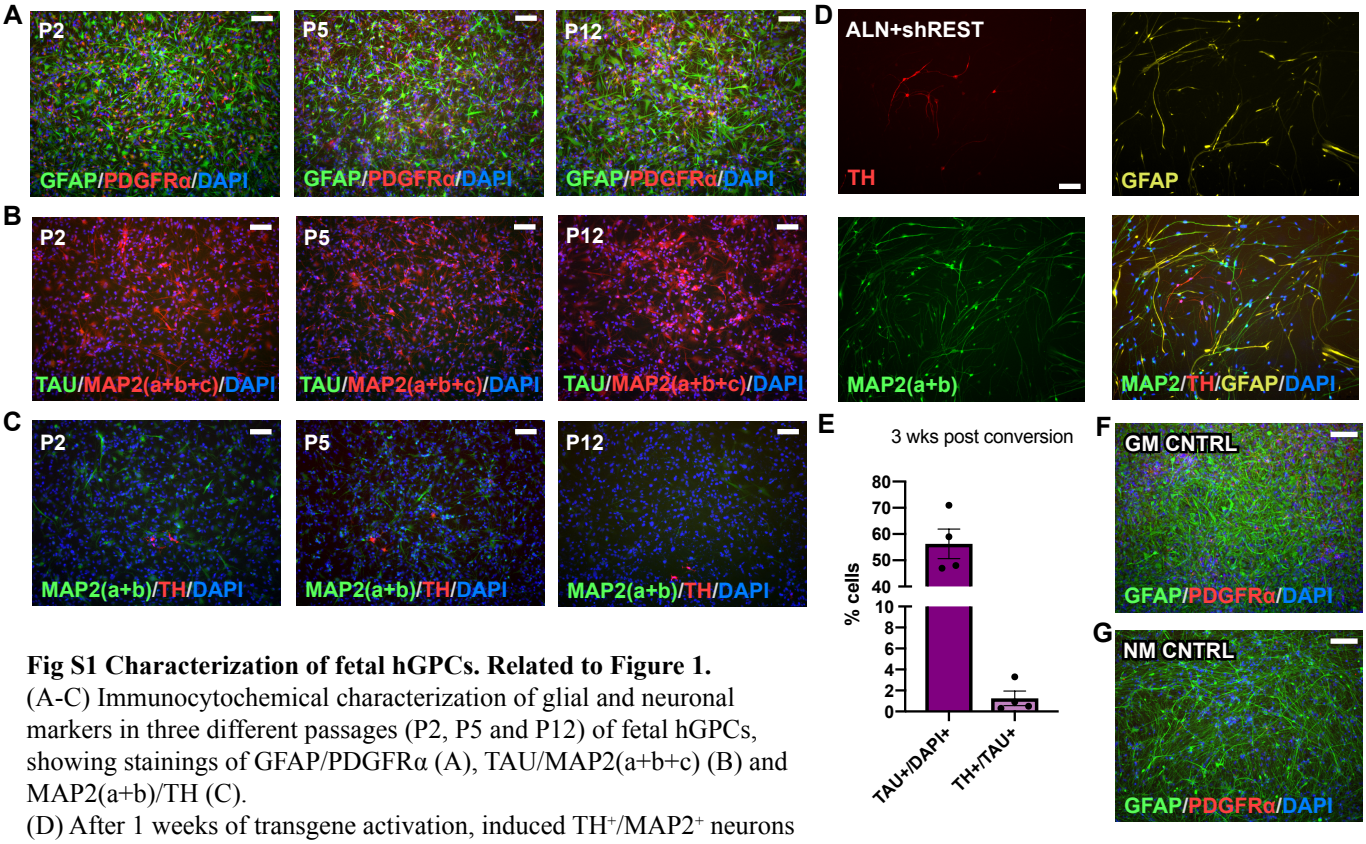

Fig S1 Characterization of fetal hGPCs. Related to Figure 1.
(A-C) Immunocytochemical characterization of glial and neuronal markers in three different passages (P2, P5 and P12) of fetal hGPCs, showing stainings of GFAP/PDGFRα (A), TAU/MAP2(a+b+c) (B) and MAP2(a+b)/TH (C).
(D) After 1 weeks of transgene activation, induced $TH^+/MAP2^+$ neurons appear in the culture and the number of $GFAP^+$ cells decrease.
(E) Quantifications of neuronal proportion (56.3 ± 5.6%) and TH^+ neuronal proportion (1.25 ± 0.69%) after three weeks of reprogramming.
(F and G) $GFAP^+$ and $PDGFR\alpha^+$ hGPCs kept in glial medium (F) or neuronal conversion medium (G) for 3 weeks in parallel to reprogrammed cells. In Fig S1E, data are presented as mean ± SEM and all data points have been visualized in the graphs. Each data point represents a replicatefrom an independent experiment. Scale bars: 100 µm.

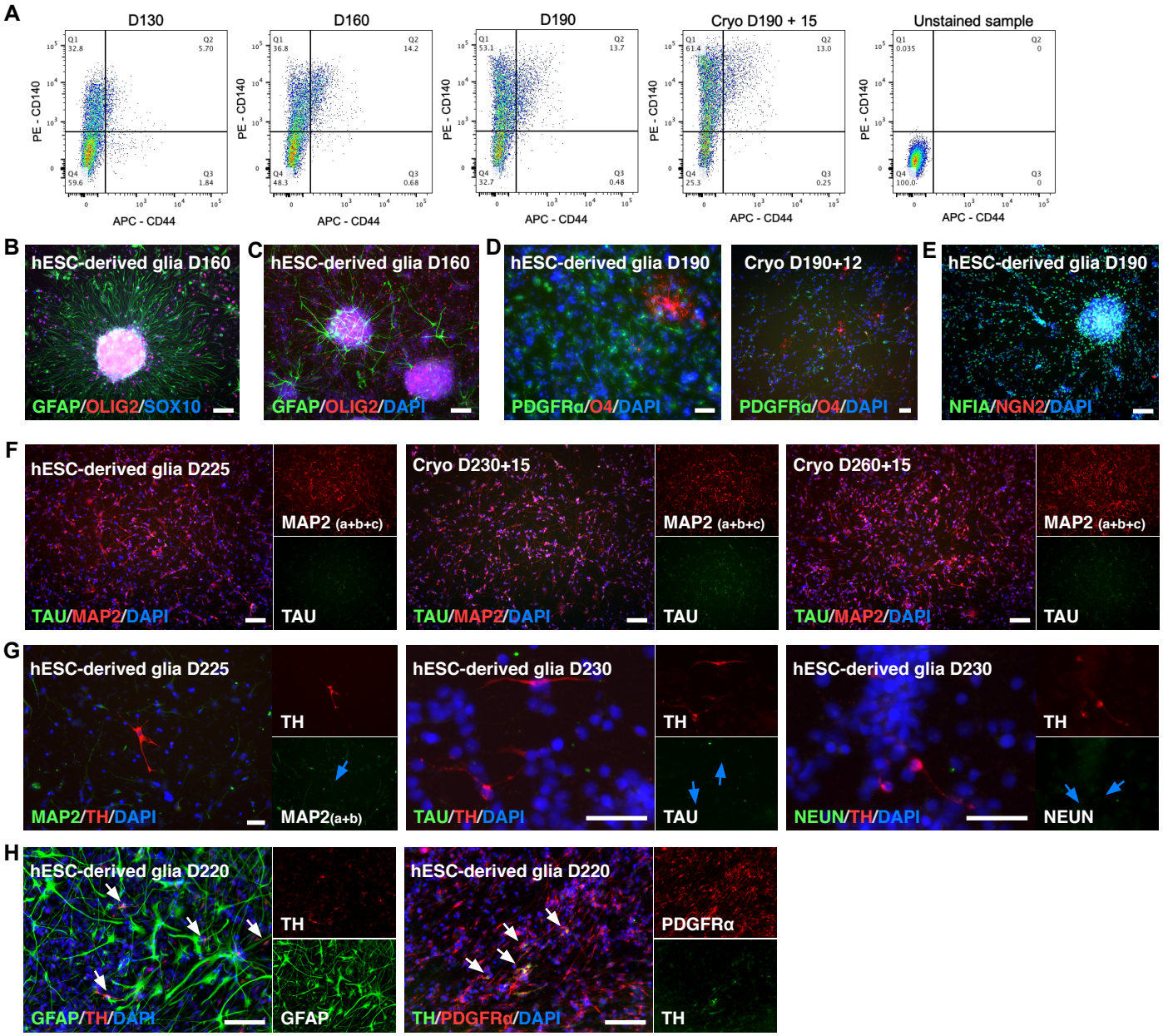

Fig S2 Characterization of hESC-derived hGPCs. Related to Figure 2.

(A) Flow cytometry analysis of the expression of CD140 and CD44 over time and following cryopreservation of hGPCs at day 190 of differentiation +15 days post thaw.

(B-E) Immunocytochemical characterization of glial markers in hESC-derived hGPCs at different stages and before and after cryopreservation shows cells positive for GFAP, OLIG2 and SOX10 (B), GFAP, OLIG2 (C), PDGFRα and O4 (D), and NFIA, but not NGN2 (E).

(F) Immunocytochemical characterization of MAP2(a+b+c) and TAU in hESC-derived hGPCs at different stages and before and after cryopreservation

(G-H) Cultures of hESC-derived hGPCs contain rare TH^+ cells. These TH^+ cells are of a glial identity since they do no not co-label with MAP2 (a+b), TAU or NEUN (G, indicated with a blue arrows), but instead co-label with GFAP and PDGFRα (H, indicated with white arrows). Scale bars: (A–C, F, H) 100 µm; (D,G) 50 µm.

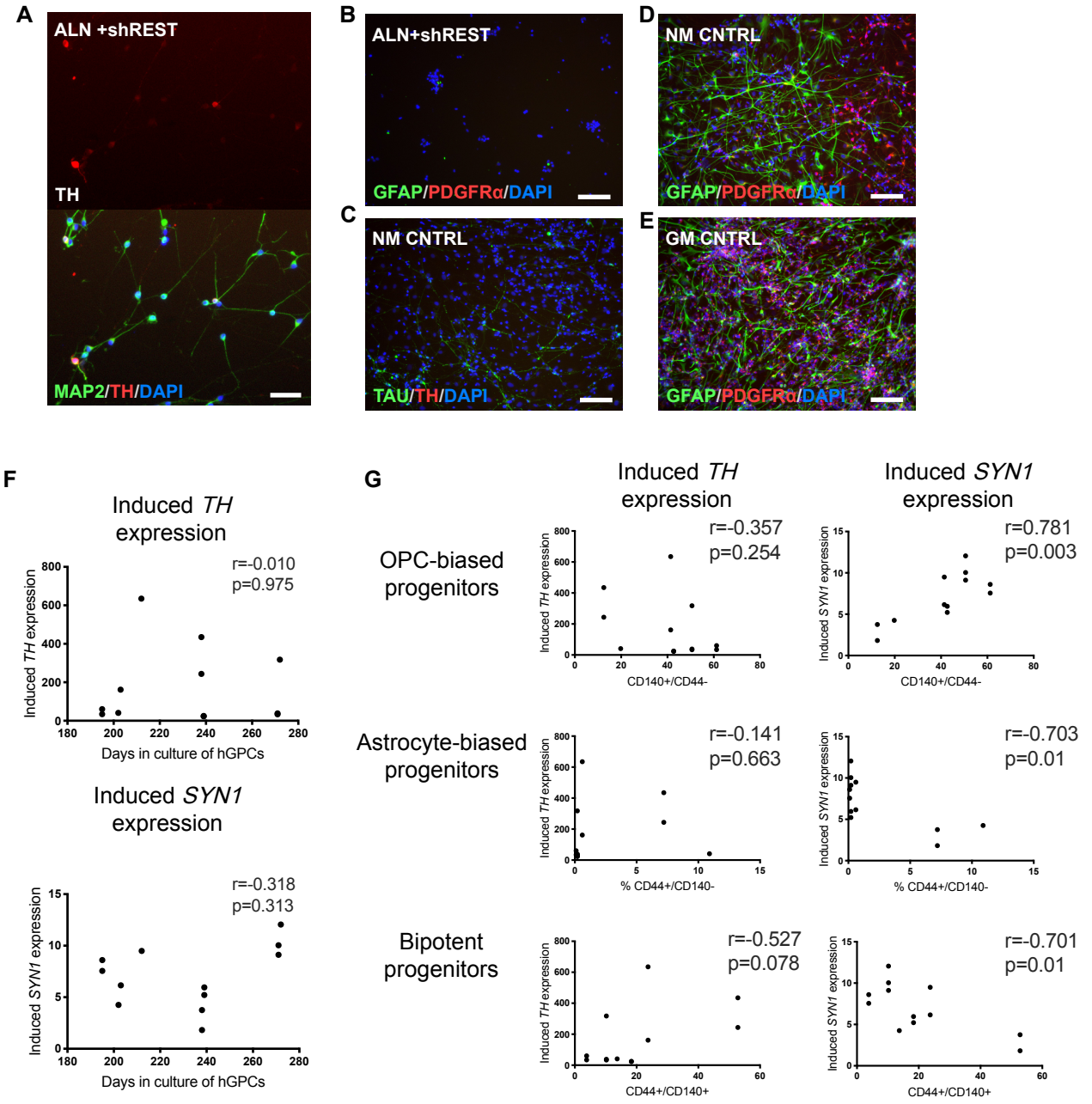

Fig S3 Direct neuronal reprogramming of hESC-derived hGPCs. Related to Figure 2.

(A) After 1 week of transgene activation, induced TH^+/$MAP2^+$ neurons appear in the culture.

(B) 3 weeks after transgene activation, $GFAP^+$ and $PDGFR\alpha^+$ cells completely disappear from the culture.

(C) hESC-derived hGPCs kept in parallel in neuronal conversion medium for 3 weeks do not give rise to any TH^+/TAU^+ neurons.

(D and E) $GFAP^+$ and $PDGFR\alpha^+$ cells are maintained when the hESC-derived hGPCs are kept in neuronal conversion medium (D) and glial medium (E).

(F) Days in culture prior to the reprogramming does not affect the induction of *TH* or *SYN1* in the reprogrammed cultures.

(G) The variable proportion of the different progenitor types in different starting cell batches affects the induction of *SYN1*, but not of *TH*, in the reprogrammed cells.

In Fig S3F and S3G, each data point represents a replicate from an independent experiment and correlations have been assessed using Pearson's r.

Scale bars: (A) 50 μm; (B-E) 100 μm.

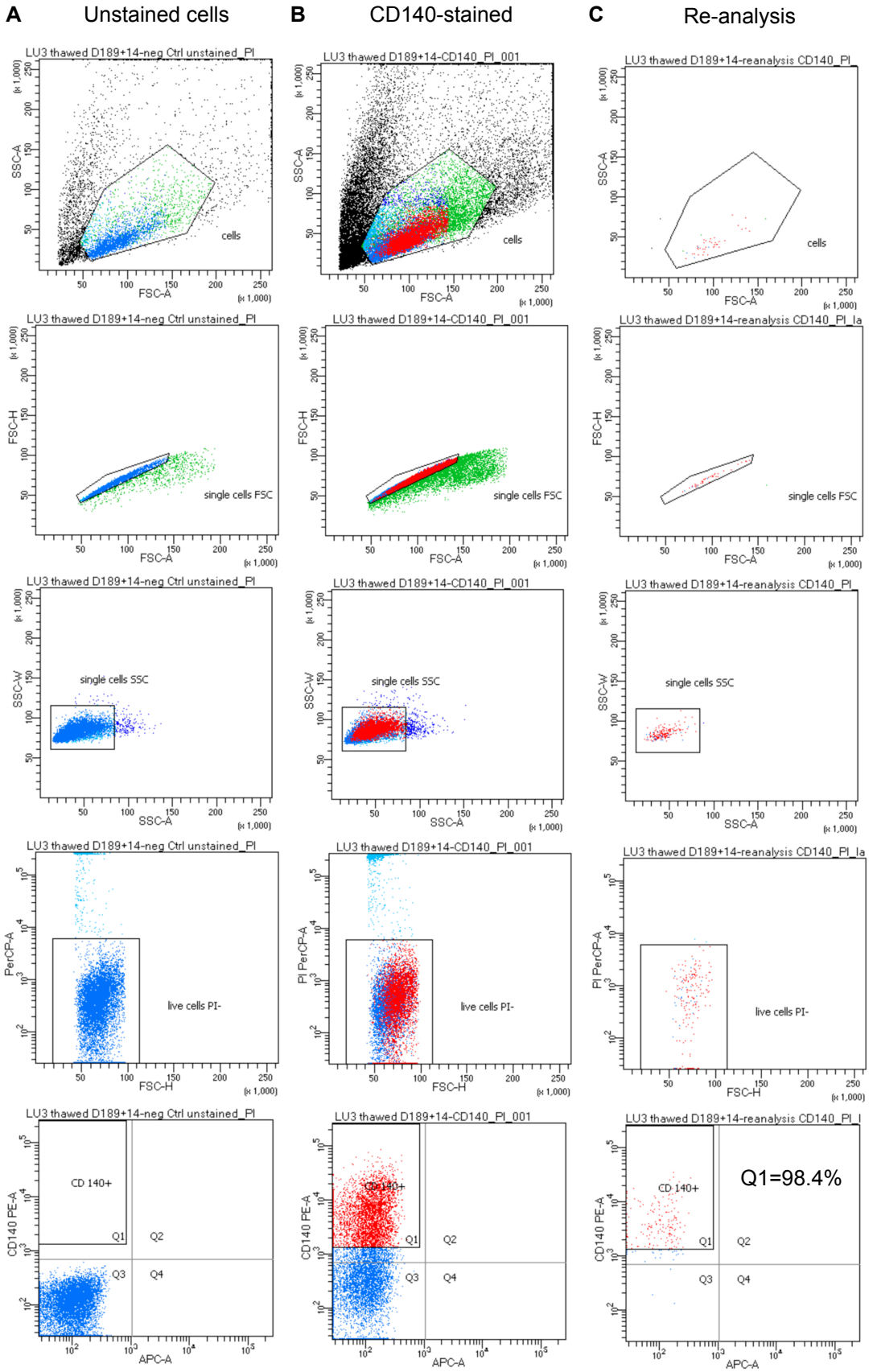

Fig S4. FACS sorting of CD140+ cells for neuronal reprogramming. Related to Figure 3.
(A-C) FACS plots showing the gating strategy for sorting CD140^{+} single cells and reanalysis plot to assess the stringency of the strategy. Unstained cells are shown in (A), CD140-stained cells are shown in (B) and re-analyzed CD140-stained cells are shown in (C).

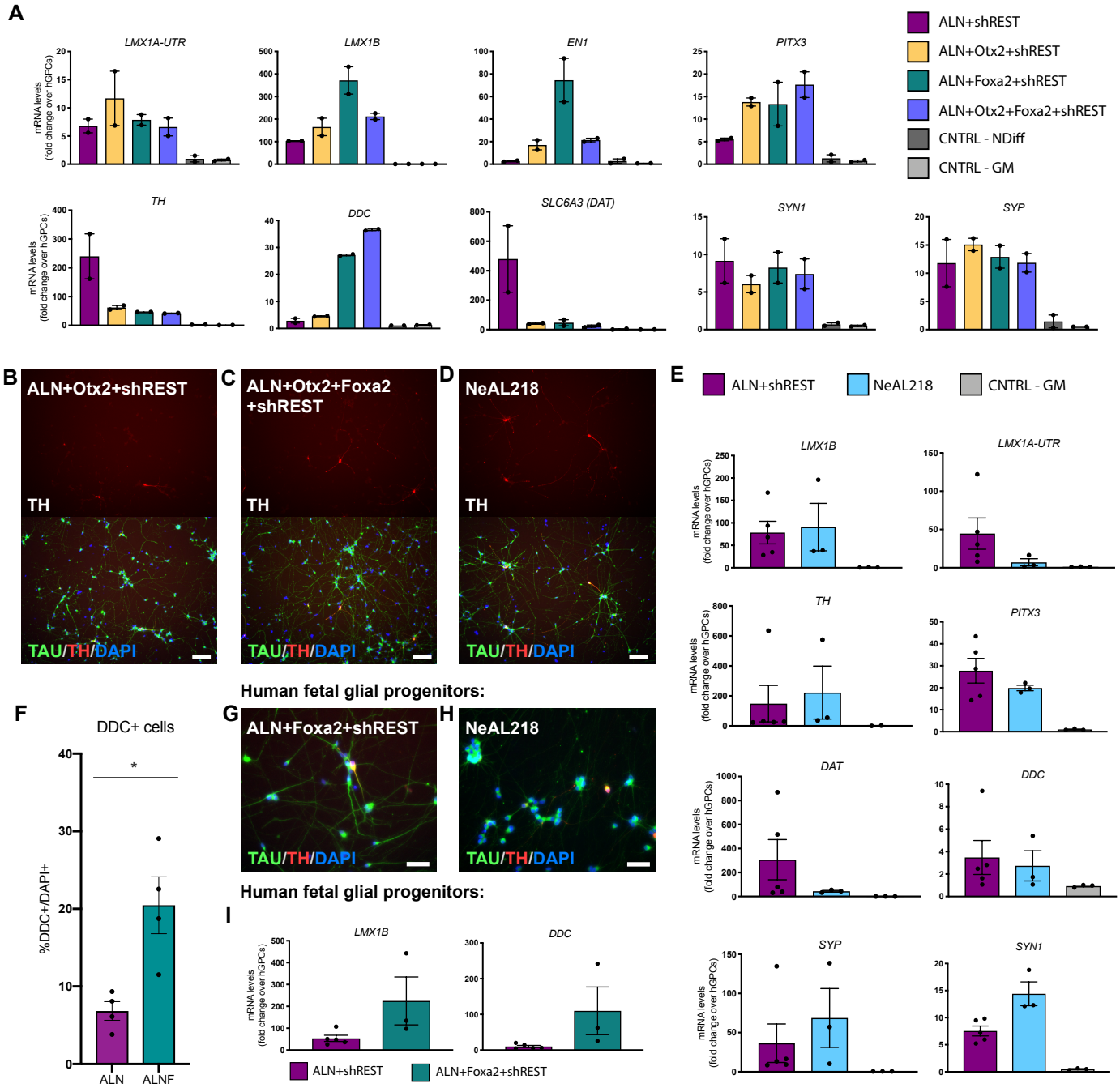

Fig S5 Assessment of factor combinations for direct conversion to a DA fate. Related to Figure 4.

(A) qPCR analysis of reprogrammed cells 3 weeks after transgene activation comparing cells reprogrammed with ALN+shREST, or with ALN+shREST together with Otx2 and/or Foxa2.

(B-D) TH$^+$/TAU$^+$ cells generated with either ALN+Otx2+shREST (B), ALN+Otx2+Foxa2+shREST (C), or NeAL218 (D) 3 weeks after transgene activation starting from hESC-derived hGPCs.

(E) qPCR analysis of reprogrammed cells 3 weeks after transgene activation comparing cells reprogrammed with either ALN+shREST or NeAL218 starting from hESC-derived hGPCs.

(F) Quantification of the proportion of DDC$^+$ cells in cultures reprogrammed with either ALN+shREST or ALN+Foxa2+shREST.

(G-H) Fetal primary hGPCs can be reprogrammed into TH$^+$/TAU$^+$ cells using ALN+Foxa2+shREST (F) and NeAL218 (G).

(I) Addition of Foxa2 to the reprogramming ALN+shREST cocktail results in a higher expression of *LMX1B* and *DDC* in fetal hGPCs.

In Fig S5A, S5E-F and S5I, data are presented as mean ± SEM and all data points have been visualized in the graphs. For the gene expression data in Fig S5A, S5E and S5I, each data point represents a replicate from an independent experiment. (S5A: n=2; S5E n=5 for ALN+shREST; n=3 for NeAL218; n=3 for CNTRL – GM; S5I: n=5 for ALN+shREST; n=3 for ALN+Foxa2+shREST). The data points displayed for qPCR data for ALN+shREST, ALN+Foxa2+shREST and CNTRLs in Fig S5A and S5E are also included of the comparison of ALN+shREST and ALN+Foxa2+shREST in Fig 4E. The data points displayed for qPCR data of *LMX1B* for ALN+shREST in Fig S5I are also incuded in Fig 1F. In Fig S5F, the proportions of DDC$^+$ cells were calculated from 446 (ALN+shREST) and 449 (ALNF+shREST) cells in 4 randomly sampled fields from one experiment. The proportions of DDC$^+$/DAPI$^+$ for the conditions ALN+shREST and ALN+FOXA2+shREST were compared using an unpaired, two-tailed, t-test; *p<0.05 (p=0.0124). Scale bars: (B-D) 100 µm; (G-H) 50 µm.

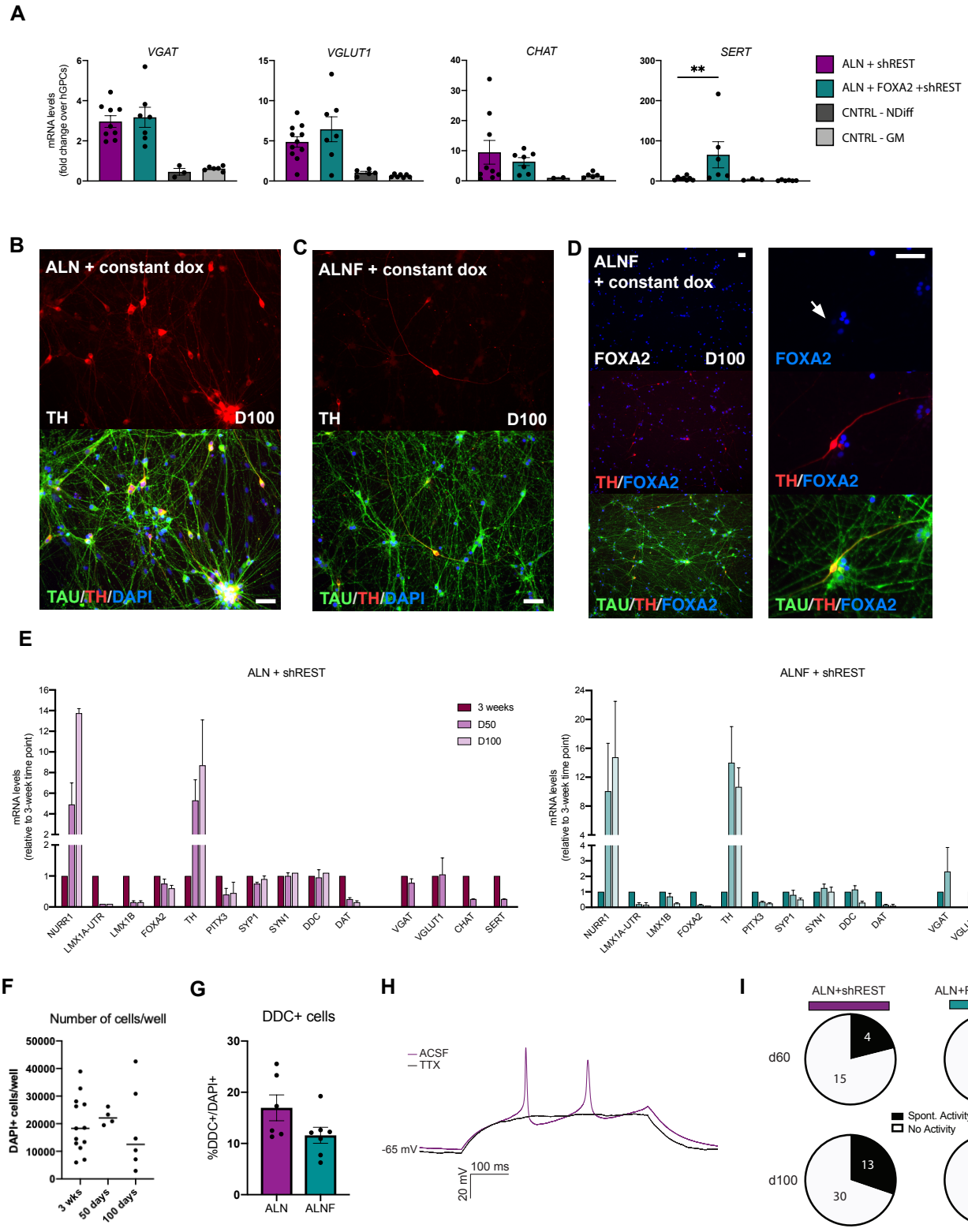
A
VGAT
VGLUT1
CHAT
SERT
mRNA levels
(fold change over hGPCs)
**
ALN + shREST
ALN + FOXA2 +shREST
CNTRL - NDiff
CNTRL - GM
B
ALN + constant dox
TH
D100
TAU/TH/DAPI
C
ALNF + constant dox
TH
D100
TAU/TH/DAPI
D
ALNF
+ constant dox
FOXA2
D100
TH/FOXA2
TAU/TH/FOXA2
FOXA2
TH/FOXA2
TAU/TH/FOXA2
E
ALN + shREST
ALNF + shREST
mRNA levels
(relative to 3-week time point)
3 weeks
D50
D100
NURR1
LMX1A-UTR
LMX1B
FOXA2
TH
PITX3
SYP1
SYN1
DDC
DAT
VGAT
VGLUT1
CHAT
SERT
F
Number of cells/well
DAPI+ cells/well
3 wks
50 days
100 days
G
DDC+ cells
%DDC+/DAPI+
ALN
ALNF
H
ACSF
TTX
-65 mV
100 ms
20 mV
I
ALN+shREST
ALN+Foxa2+shREST
d60
d100
4
15
3
9
13
30
10
31
Spont. Activity
No Activity

Fig S6 Phenotypic maturation of iDANs. Related to Figures 5 and 6.

(A) qPCR analysis of different neuronal markers in ALN+shREST and ALN+Foxa2+shREST reprogrammed cells 3 weeks after transgene activation.

(B-C) Maintained doxycycline administration does not affect the proportion of TH^+ iDANs when cells are reprogrammed with ALN+shREST (B) but results in a lower proportion on TH^+ neurons when the cells are reprogrammed with ALN+Foxa2+shREST (C).

(D) Cells that show a strong labeling of FOXA2 are not TH^+. White arrow in right panel indicates a TH^+ cell with weak nuclear labeling of FOXA2.

(E) Gene expression analysis at late timepoint relative to the expression of each gene at the early 3-week timepoint.

(F) Quantification of the proportion of DDC^+ cells in cultures reprogrammed with either ALN+shREST or ALN+Foxa2+shREST after 100 days.

(G) Quantifications of the numbers of cells per well in repgrogrammed cutures after 3 weeks, 50 days and 100 days.

(H) Representative trace of voltage responses from whole cell patch clamp technique showing induced AP (purple) selectively blocked by tetrodotoxin (TTX, black).

(I) Pie chart showing different number of cells exhibiting spontaneous postsynaptic activity at 60 and 100 days after reprogramming start with ALN+shREST (left) and ALN+Foxa2+shREST (right).

Data are presented as mean ± SEM. In Fig S6A, each data point represents a replicate from an independent experiment (n=9-11 for ALN+shREST; n=7 for ALN+Foxa2+shREST; n=3-5 for CNTRL – NDIFF; n=6-8 for CNTRL – GM). The gene expression levels for the conditions ALN+shREST and ALN+Foxa2+shREST were compared using a Mann-Whitney test; $^{**}p<0.01$ ($p=0.0079$ for SERT). In Fig S6E gene expression data was collected from 2 independent experimental replicates per time point and the expression is described relative to the early time point. In Fig S6G, the proportions of DDC^+ cells were calculated from 717 (ALN+shREST) and 1,249 (ALN+Foxa2+shREST) cells in 6-7 randomly sampled fields from one experiment. The proportions of $DDC^+/DAPI^+$ for the conditions ALN+shREST and ALN+Foxa2+shREST were compared using an unpaired, two-tailed, t-test ($p=0.0901$). Scale bars: 50 μm.

Table S1: Summary of electrophysiological properties of fetal hGPCs. Related to Figure 1.

Intrinsic Properties	ALN + shREST
Resting membrane potential (mV)	d80-120= -30.41 ± 1.51
Cell capacitance (pF)	d80-120 = 17.94 ± 1.42

Table S2: Cell batches used to as starting cells for reprogramming experiments. Related to Figure 2.

hESC-line	hGPCs Batch ID	Days in culture	Total $CD140^+$ cells	Total $CD44^+$ cells	$CD140^+/CD44^+$	$CD140^+/CD44^-$	$CD44^+/CD140^-$
RC17	LU7	165	64.9%	14.7%	8.7%	48.9%	3.7%
RC17	LU3	203	65.2%	28.6%	23.7%	41.4%	0.6%
RC17	LU3	212	65.2%	28.6%	23.7%	41.4%	0.6%
RC17	LU13	238	59.9%	50.8%	52.8%	12.5%	7.2%
RC17	LU16	202	32.4%	23.8%	13.7%	19.8%	10.9%
HS1001	LU27	195	66.7%	3.2%	3.8%	61.2%	0.1%
RC17	LU3	239	60.9%	20.1%	18.3%	42.7%	0.2%
RC17	LU6	271	60.5%	10.0%	10.2%	50.6%	0.2%

Table S3: Summary of electrophysiological properties of hESC derived hGPC. Related to Figure 6.

Intrinsic Properties	ALN	ALNF	P (between groups)
Resting membrane potential (mV)	d60 = -28.64 ± 1.2 d100 = -28.14 ± 1.1	d60 = -25.58 ± 1.5 d100 = -32.88 ± 0.8	>0.999 0.086
Cell capacitance (pF)	d60 = 14.81 ± 1.3 d100 = 14.46 ± 1.2	d60 = 10.36 ± 1.0 d100 = 14.78 ± 1.1	0.471 >0.999
Input resistance (MΩ)	d60 = 3469 ± 476.0 d100 = 3347 ± 207.6	D60 = 4928 ± 402.2 D100 = 3476 ± 377.6	0.247 >0.999

There were no significant differences between the groups or times post-conversion (d60 vs d100) in any of the intrinsic properties. mV = millivolt, pF = picofarad, MΩ = Mega Ohm. One-way ANOVA with Bonferroni multiple comparison post hoc test was used for statistical comparison.

Table S4: Primary antibodies used in this study. Related to Figure 1-5.

Antigen	Species	Company (cat. no.)	Dilution
ALDH1A1	Rabbit	Abcam (ab24343)	1:200
DDC	Rabbit	Merck Millipore (AB1569)	1:500
FOXA2	Goat	R&D systems (AF2400)	1:500
GFAP	Mouse	Biolegend (SMI 21)	1:500
MAP2	Mouse	Sigma Aldrich (M1406)	1:500
MAP2	Rabbit	Millipore (AB5622)	1:500
NEUN	Rabbit	Millipore (ABN78)	1:500
OLIG2	Rabbit	Neuromics (RA25081)	1:500
O4	Mouse	Merck Millipore (MAB345)	1:100
PDGFRα	Rabbit	Cell Signaling Technology (5241S)	1:300
SOX10	Goat	R&D systems (AF2864)	1:25
SYNAPSIN 1	Rabbit	Merck Millipore (AB1543)	1:200
TAU (HT7)	Mouse	Thermo Fisher (MN1000)	1:500
TH	Rabbit	Merck Millipore (AB152)	1:1,000
TH	Sheep	Merck Millipore (AB1542)	1:1,000
VIMENTIN	Chicken	Merck Millipore (AB5733)	1:2,000
VMAT2	Rabbit	Sigma Aldrich (AB1598P)	1:200

Table S5: Primers used for qRT-PCR analysis. Related to Figure 1 and 4.

Gene	Full gene name	Primer sequence (fwd/rev)
ACTB	Beta-actin	CCTTGCACATGCCGGAG
		GCACAGAGCCTCGCCTT
CHAT	Choline O-acetyltransferase	TCCAACGAGGACGAGCGTTTGC
		CGAGTCCCGGTTGGTGGAGTCT
DDC	DOPA decarboxylase	GGGGACCACAACATGCTGCTCC
		AATGCACTGCCTGCGTAGGCTG
DAT (SLC6A3)	Dopamine transporter	CACTGCAACAACTCCTGGAA
		AAGTACTCGGCAGCAGGTGT
EN1	Engrailed 1	CGTGGCTTACTCCCCATTTA
		TCTCGCTGTCTCTCCCTCTC
FOXA2	Forkhead box A2	CCGTTCTCCATCAACAACCT
		GGGGTAGTGCATCACCTGTT
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TTGAGGTCAATGAAGGGGTC
		GAAGGTGAAGGTCGGAGTCA
GFAP	Glial fibrillary acidic protein	TCATCGCTCAGGAGGTCCTT
		CTGTTGCCAGAGATGGAGGTT
LMX1A (UTR)	LIM homeobox transcription factor a	CGCATCGTTTCTTCTCCTCT
		CAGACAGACTTGGGGCTCAC
LMX1B	LIM homeobox transcription factor b	CTTAACCAGCCTCAGCGACT
		TCAGGAGGCGAAGTAGGAAC
NCAM1	Neural cell adhesion molecule 1	GTCAGAGGCCACCGTCAACGTG
		CTTCCCCCTCCCGGAACTCCTG
NR4A2 (NURR1, intron-spanning)	Nuclear receptor subfamily 4, group A, member 2	CAGGCGTTTTCGAGGAAAT
		GAGACGCGGAGAACTCCTAA
OTX2	Orthodenticle homeobox 2	ACAAGTGGCCAATTCACTCC
		GAGGTGGACAAGGGATCTGA
PDGFRA	Platelet derived growth factor receptor alpha	CCTTGGTGGCACCCCTTAC
		TCCGGTACCCACTCTTGATCTT
PITX3	Paired-like homeodomain 3	GGAGGTGTACCCCGGCTACTCG
		GAAGCCAGAGGCCCCACGTTGA
SERT (SLC6A4)	Serotonin transporter	TGGACCCTCCATTCCACGTCCC
		GTCCTGGAGCCCCTTAGACCGG
SYP	Synaptophysin	ACCTCGGGACTCAACACCTCGG
		GAACCACAGGTTGCCGACCCAG
SYN1	Synapsin 1	CCCGTGGTTGTGAAGATGGGGC
		TGCCACGACACTTGCGATGTCC
TH	Tyrosine hydroxylase	CGGGCTTCTCGGACCAGGTGTA
		CTCCTCGGCGGTGTACTCCACA
VGAT (SLC32A1)	Vesicular inhibitory amino acid transporter	AGATGATGAGAAACAACCCCAG
		CACGACAAGCCCAAAATCAC
VGLUT1 (SLC17A7)	Vesicular glutamate transporter 1	AATAACAGCACGACCCACCGCG
		AGCCGTGTATGAGGCCGACAGT

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of human GPCs from hESCs

To initiate the differentiation, the hESCs were detached from the plate using accutase and dissociated to single cells and small cell clusters and kept in iPS-Brew XF medium on day 0 in ultra-low adhesion flasks to allow for embryoid body (EB) formation. On day 0 – day 1, the iPS-Brew XF medium was supplemented with 10 µM Y-27632 (ROCK inhibitor; Miltenyi) to improve the survival of single cells. On day 5, the medium was switched to neural induction medium (NIM), containing DMEM/F12 basal medium (Thermo Fisher), MEM Non-Essential Amino Acids (NEAA) Solution (Thermo Fisher), N2 supplement (Thermo Fisher), and Antibiotic-Antimycotic (Thermo Fisher), and supplemented with bFGF (20 ng/ml, Sigma-Aldrich) and Heparin (2 µg/ml, STEMCELL Technologies). On day 9, the EBs were attached on Poly-Ornithine/Laminin (PO/Lam) coated tissue culture plates and two days later the medium was changed to NIM + RA (0.1 µM, Sigma-Aldrich) and on day 16 the medium was changed to NIM/B27 medium + RA + Purmorphamine (1 µM, Millipore). Around day 26-29, the cells were detached from the plates and were seeded as clusters in ultra-low attachment plates. At this stage, the cell quality was assessed by ICC of PAX6, SOX1 and OLIG2 and by FC of CD133 and SSEA4. Following the detachment, the free-floating cell clusters were kept in NIM/B27 medium + Purmorphamine + bFGF (10 ng/ml) up until day 37.

On day 37, the medium was changed to glial medium (GM) containing DMEM/F12 basal medium, B27 supplement, N1 supplement (Sigma-Aldrich), MEM NEAA, Antibiotic-Antimycotic, T3 (60 ng/ml, Sigma-Aldrich), db-cAMP (1 µM, Sigma-Aldrich), Biotin (100 ng/ml, Sigma-Aldrich), recombinant human PDGF-AA protein (10 ng/ml, R&D Systems), recombinant human IGF-I (10 ng/ml, R&D Systems) and recombinant human NT-3 Protein (10 ng/ml, R&D Systems), and supplemented with Purmorphamine until day 55 when Purmorphamine was withdrawn from the medium. The cells were then kept in GM for the remaining time in culture. Around day 70, the free-floating clusters were manually cut into smaller pieces using disposable sterile scalpels under a dissection microscope and seeded onto PO/Lam coated tissue culture plates. Thereafter, the cells were passaged and expanded every 30 days, when the clusters were mechanically detached from the plates using a cell scraper, cut at the dissection microscope and re-seeded again at lower density. At every passage, the cells were analyzed by FC for CD44 and CD140 and by ICC for various glial markers. Table S1 specifies the number of days in culture and the proportion of $CD140^+/CD44^+$ of the hESC-derived cell batches used in this study.

Cryopreservation of hESC-derived hGPCs

For cryopreservation of the hESC-derived GPCs, the cell clusters were detached from the plate using a cell scraper, centrifuged at 300 x g for 7 min, resuspended in cold glial medium and dissociated using a 1000 µl pipette. The cells were cryopreserved at a density 0.5-1x 6-well per cryovial in 500 µl GM. The freezing medium was prepared by supplementing ProFreezeTM-CDM NAO Freezing Medium (2X, Lonza) with 15 % DMSO and was added dropwise to the cryovial containing the cell suspension at equal proportions (giving a final concentration of DMSO of 7.5 %). The cryovials were then moved into a controlled-rate alcohol-free cell freezing container and kept at -80°C overnight. The following day the cells were moved to a -150°C freezer or a Liquid Nitrogen tank for long-term storage.

Electrophysiology

Whole cell patch-clamp electrophysiological recordings were performed at day 60 and 100 post-conversion. Cells were cultured on glass coverslips and transferred to a recording chamber with constant flow of Krebs solution gassed with 95 % O_2 – 5 % CO_2 at room temperature. The composition of the standard solution was (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 25 Glucose and 26 $NaHCO_3$. For recordings Multiclamp 700B amplifier (Molecular Devices) was used together with borosilicate glass pipettes (3–7 MOhm) filled with the following intracellular solution (in mM): 122.5 potassium gluconate, 12.5 KCl, 0.2 EGTA, 10 Hepes, 2 MgATP, 0.3 Na_3GTP and 8 NaCl adjusted to pH 7.3 with KOH as in (Pfisterer et al., 2011). Data acquisition was performed with pClamp 10.2 (Molecular Devices); current was filtered at 0.1 kHz and digitized at 2kHz. The cells were selected for recording based on their neuronal shape and clear surface, cells with signs of shrinkage or visualized nuclei were excluded. Resting membrane potentials were monitored immediately after breaking-in in current-clamp mode. Thereafter, cells were kept at a membrane potential of -60mV to -70mV, and 500ms currents were injected from -20pA to +90pA with 5pA increments to induce action potentials. For inward sodium and delayed rectifying potassium current measurements cells were clamped at -70mV and voltage-depolarizing steps were delivered for 100ms at 10mV increments. Spontaneous AP were recorded in current-clamp mode at resting membrane potentials. Addition of tetrodotoxin (TTX) to the Krebs solution to final concentration of 1 µM was performed in order to specifically block sodium channels and inhibit neuronal activity during recording.

Flow cytometry analysis and FACS sorting

For flow cytometry analysis of the cells between D130-270, the cell clusters were dissociated for 8 min in Accutase (StemPro; Thermo Fisher) and resuspended to 1 million cell/ml in Miltenyi wash buffer (PBS + 0.5% BSA Fraction V + 2 μM EDTA + 0.05‰ Phenol Red). For each staining, 100 ul cell suspension was used and the cells were incubated with fluorophore-conjugated antibodies for 15 min at 4°C (PE anti-human CD140a, BD Biosciences, cat. no. 556002, 1:10; APC anti-CD44 (Miltenyi, cat. no. 130-095-177, 1:500); APC anti-human CD133/1, (Miltenyi, cat. no. 130-113-668, 1:50); FITC anti-human SSEA-4, Biolegends, cat. no. 330410, 1:20). Following the 15 min incubation with the antibodies, the cells were washed with Miltenyi wash buffer, centrifuged for 10 min at 200 x g and transferred to 5 ml polystyrene tubes with cell-strainer caps at a final density of around 400,000 cells/ml in DMEM/F12 + DNase. To exclude dead cells, Propidium iodide (PI, Miltenyi, cat. no. 130-095-177, 1:500) was added to the samples. For each sample, 10,000 cells were analyzed on a FACSAria III sorter (BD Biosciences). Gates were set based on Fluorescence Minus One (FMO) controls and compensation was performed using single-stained cells.

For CD140-based cell sorting, the same staining procedure was employed but the cells were stained at a higher density, with the same concentration of CD140a antibody (1:10). To improve the viability of the sorted cells, the sorting medium was supplemented with 10 μM Y-27632 and polypropylene tubes that had been precoated with 3.4% BSA Fraction V in PBS overnight were used. The sorted cells were collected in glial medium supplemented with 1x B27 and 10 μM Y-27632. For gating strategy for the FACS exeperiment, see Figure S4.

Microscopy

Fluorescent images were captured using a Leica DMI6000B widefield microscope. The image acquisition software was Leica LAS X and images were processed using Adobe Photoshop CC 2018. Any adjustments were applied equally across the entire image, and without the loss of any information.