Identification of Adeno-associated virus variants for gene transfer into human neural cell types by parallel capsid screening

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Supplementary information

Supplementary Methods

Cell culture

Neural stem cells

Four different populations of human NSCs were included in this study, representing different developmental stages. Directly converted, blood-derived early stage iNSCs[20] were cultured on Matrigel-coated dishes (Corning, Corning, USA, 1:60) in 2x N2B27 (1:1 Advanced DMEM/F12 : Neurobasal (both Thermo Fisher Scientific, Waltham, USA) with 1x N2 supplement (GE Healthcare, Chicago, USA), 1x B27 supplement without vitamin A, 1 mM L-glutamine and 0.0025% BSA (all Thermo Fisher Scientific)) supplemented with 3 µM CHIR99021, 0.5 µM purmorphamine (both Miltenyi, Bergisch Gladbach, Germany), 0.5 µM A83-01 (Tocris, Bristol, UK), 10 ng/ml human LIF (Novoprotein, Fremont, USA) and 64 µg/ml LAAP (Sigma-Aldrich, St. Louis, USA). Early stage iPSC-derived smNPCs[21] were grown on Geltrex-coated dishes (Thermo Fisher Scientific, 1:50) in 1x N2B27 (1:1 DMEM/F12 (Thermo Fisher Scientific) : Neurobasal with 0.5x N2 supplement, 0.5x B27 supplement without vitamin A, 1 mM L-glutamine and 1x Pen/Strep (Thermo Fisher Scientific)) supplemented with 3 µM CHIR99021, 0.5 µM purmorphamine and 64 µg/ml LAAP. For virus screening, $1x10^4$ cells were seeded in 200 µl of their respective medium per well of a 96-well ibidi imaging plate (Gräfelfing, Germany). For assessment of proliferative and differentiated iNSCs, cells were seeded at a density of $5x10^4$ cells/cm^2 and $9x10^4$ cells/cm^2, respectively. For gain-of-function experiments with iNSCs, spontaneous differentiation was induced by switching culture medium to NGMC at the day of AAV transduction (1:1 DMEM/F12 : Neurobasal with 0.5x N2 supplement, 0.5x B27 supplement without vitamin A, 800 µg/ml D(+)glucose (Carl Roth, Karlsruhe, Germany), 0.5 mM L-glutamine and 1x Pen/Strep, supplemented with 10 ng/ml BDNF, 10 ng/ml GDNF (both Cell Guidance Systems, Cambridge, UK), 64 µg/ml LAAP and 0.5 mM dibutyryl-cAMP (Sigma-Aldrich)).

Polarized ltNES[22] were cultured in N2 medium (DMEM/F12 with 20 nM progesterone, 100 µM putrescin, 30 nM sodium selenite, 100 µg/ml apo-transferrin (all Sigma-Aldrich), 25 µg/ml insulin (Thermo Fisher Scientific) and 1.6 mg/ml D(+)-glucose) supplemented with 0.05x B27 supplement (Thermo Fisher Scientific), 10 ng/ml EGF and 10 ng/ml bFGF (both R&D systems, Minneapolis, USA) on poly-L-ornithine-laminin (Sigma-Aldrich)-coated dishes. $1.3x10^5$ ltNES were seeded in 200 µl medium per well in a 96-well plate for AAV screening.

Later stage iPSC-derived gliogenic RGL-NPCs[23] were cultured on Geltrex-coated dishes (1:50) in N2 medium (DMEM/F12 with 1x N2 supplement, 1.6 mg/ml D(+)-glucose, 25 µg/ml insulin and 1x

Pen/Strep) supplemented with 20 ng/ml EGF and 20 ng/ml bFGF. For virus screening, $0.7x10^4$ RGL-NPCs were seeded in 200 µl medium per well in a 96-well plate.

Neurons

Three different types of iPSC-derived neuronal cultures were subjected to virus screening. LtNES were differentiated into neuroglial cultures by withdrawing the growth factors EGF and bFGF. Cells were differentiated in 1:1 DMEM/F12 : Neurobasal with 0.5x N2 supplement, 0.5x B27 supplement, 0.9 mg/ml D(+)-glucose, 10 µg/ml insulin and 1x Anti-Anti (Thermo Fisher Scientific), and cryo-preserved on day 14 of differentiation. For experiments, pre-differentiated cells were thawed in ltNES differentiation medium supplemented with 10 ng/ml BDNF and 10 ng/ml GDNF, seeded at a density of $2.5x10^4$ cells per well for virus screening and $5.5x10^4$ cells/cm^2 for cell type characterization, and further matured up until day 27 of differentiation. Alternatively, iPSCs were differentiated into neurons via overexpression of the transcription factor(s) NGN2 or ASCL1 plus DLX2 according to a previously published protocol[24]. On day 8 of forward programming, cells were replated and seeded at a density of $4.5x10^4$ cells per well. One day after seeding, transduction of the AAV screening panel was performed. For all neuronal cultures, AAV panel transduction was performed in 200 µl medium per well in a 96-well plate.

RGL-NPCs and astrocytes

In order to derive highly purified astrocyte cultures, RGL-NPCs were seeded at a density of $9x10^4$ cells/cm^2 on Matrigel-coated dishes (1:30). Differentiation was initiated by switching to N2 medium supplemented with 100 µg/ml apo-transferrin, 10 ng/ml human LIF and 10 ng/ml BMP-4 (Thermo Fisher Scientific). For virus screening, cells were replated on day 28 of astrocyte differentiation, by seeding $0.7x10^4$ astrocytes per well. Virus screening was performed on days 29-31 of differentiation in 200 µl medium. For functional experiments, astrocytes were seeded at a density of $9x10^4$ cells/cm^2 on day 14 of astrocyte differentiation and AAVs were transduced one week later. Upon AAV transduction, medium was changed to N2 medium (1x DMEM/F12 with 1x N2 supplement, 1.6 mg/ml D(+)glucose, 50 µg/ml insulin, 100 µg/ml apo-transferrin and 1x Pen/Strep) supplemented with 1x B27 supplement without vitamin A, 10 ng/ml BDNF and 10 µM Rock-inhibitor Y-27632 (Cell Guidance Systems), in order to support the survival of astrocytes as well as eventually emerging neurons.

iPSC-derived microglia

To generate human iPSdMiG, iPSCs were cultured on Geltrex-coated 6-well plates (180 µg/ml) in iPS-brew (Stemcell Technologies, Vancouver, Canada) medium and passaged using 0.5 mM EDTA (Sigma-Aldrich). To induce differentiation, embryoid bodies were generated by detaching intact iPSC colonies

using 1 mg/ml collagenase (Thermo Fisher Scientific) dissolved in DMEM/F12. Detached colonies were cultured in suspension for four days before being seeded on poly-L-ornithine- plus fibronectin (Sigma-Aldrich)-coated culture plates. The differentiation of iPSdMiG was carried out according to a proprietary protocol of the LIFE & BRAIN GmbH (patent application number EP20162230). In short, neuroepithelial and hemogenic endothelial precursors were differentiated into neural and immature microglial cells within the same culture paradigm. After approximately six weeks of differentiation, mature microglia were released into the supernatant of the multilineage differentiation culture, from which they could be repeatedly harvested and plated in poly-L-lysine (Sigma-Aldrich)-coated cell culture plates in order to obtain homogeneous, adherent cultures. For AAV screening, $2.3x10^3$ harvested iPSdMiG were seeded per well in 200 µl of their respective medium.

Supplementary Tables

Supplementary Table S1: Overview of peptide modifications inserted into the capsids of diverse AAV serotypes. Table adapted from Boerner *et al.*, 2020[19].

Peptide	Source Reference	DNA sequence [a]	Protein sequence
P1	54	CGC GGC GAT CTG GGC CTG AGC	RGDLGLS
P2	26	TGC GAT TGC CGC GGC GAT TGC TTT TGC	CDCRGDCFC
P4	55	AAC GAT GTG CGC AGC GCG AAC	NDVRSAN
P5	55	AAC GAT GTG CGC GCG GTG AGC	NDVRAVS
A1	19	ATG CCA TTA GGA GCG GCA GGC	MPLGAAG
A2	19	AAC TAC TCC AGA GGA GTG GAC	NYSRGVD
A6	19	AAC GAG GCG CGG GTC CGG GAG	NEARVRE

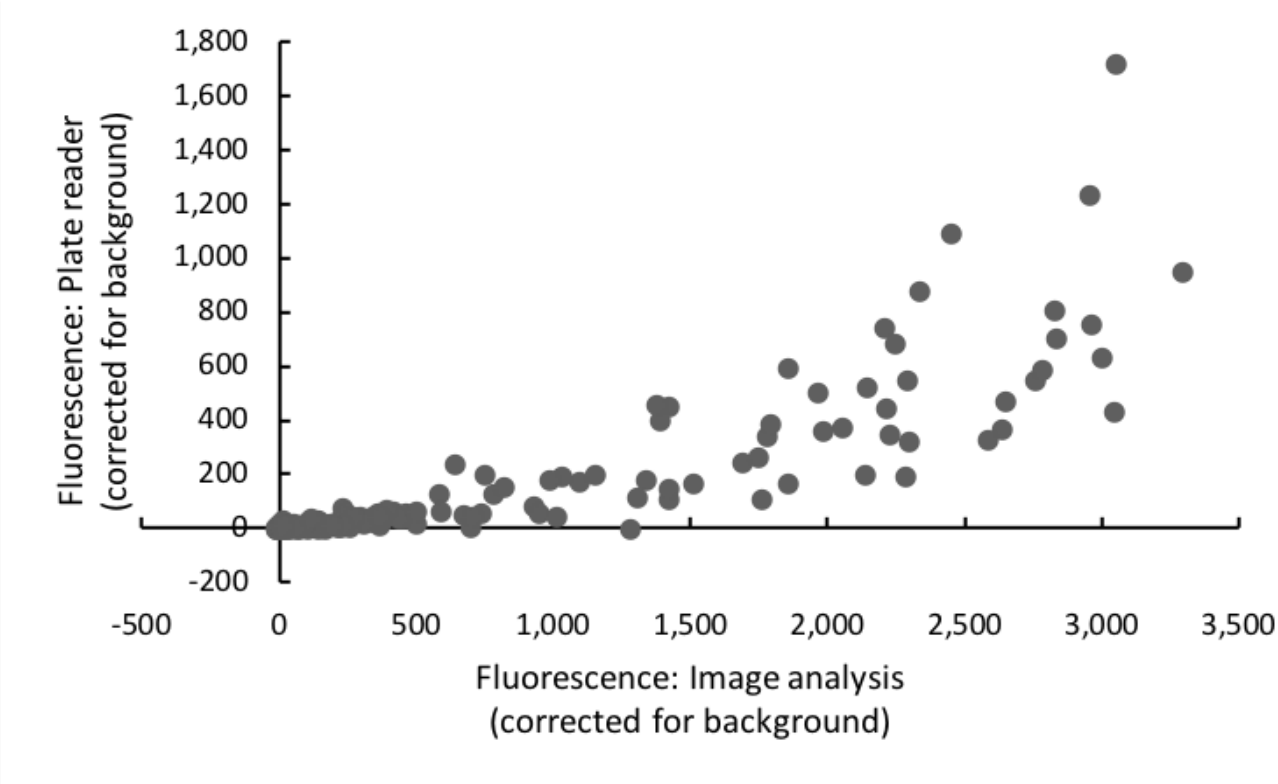
[a] *Forward oligonucleotides encoding for the peptide.*

Supplementary Table S2: Peptide insertion sites in the different AAV serotypes screened. Table adapted from Boerner *et al.*, 2020[19].

Capsid	Insertion site [a]
AAV1	(i) D590_P591
	(ii) S588_T589
AAV2	(i) R588_Q589
	(ii) N587_R588
AAV3	(i) S586_S587
	(ii) N588_T589
AAV4	(i) S584_N585
	(ii) S586_N587
AAV5	(i) S575_S576
	(ii) T577_T578
AAV6	(i) D590_P591
	(ii) S588_T589
AAV7	N589_T590
AAV8	N590_T591
AAV9	Q588_A589
AAVrh.10	N590_A591
AAVpo1	(i) N567_S568
	(ii) N569_T570
AAV12	(i) N592_A593
	(ii) T594_T595

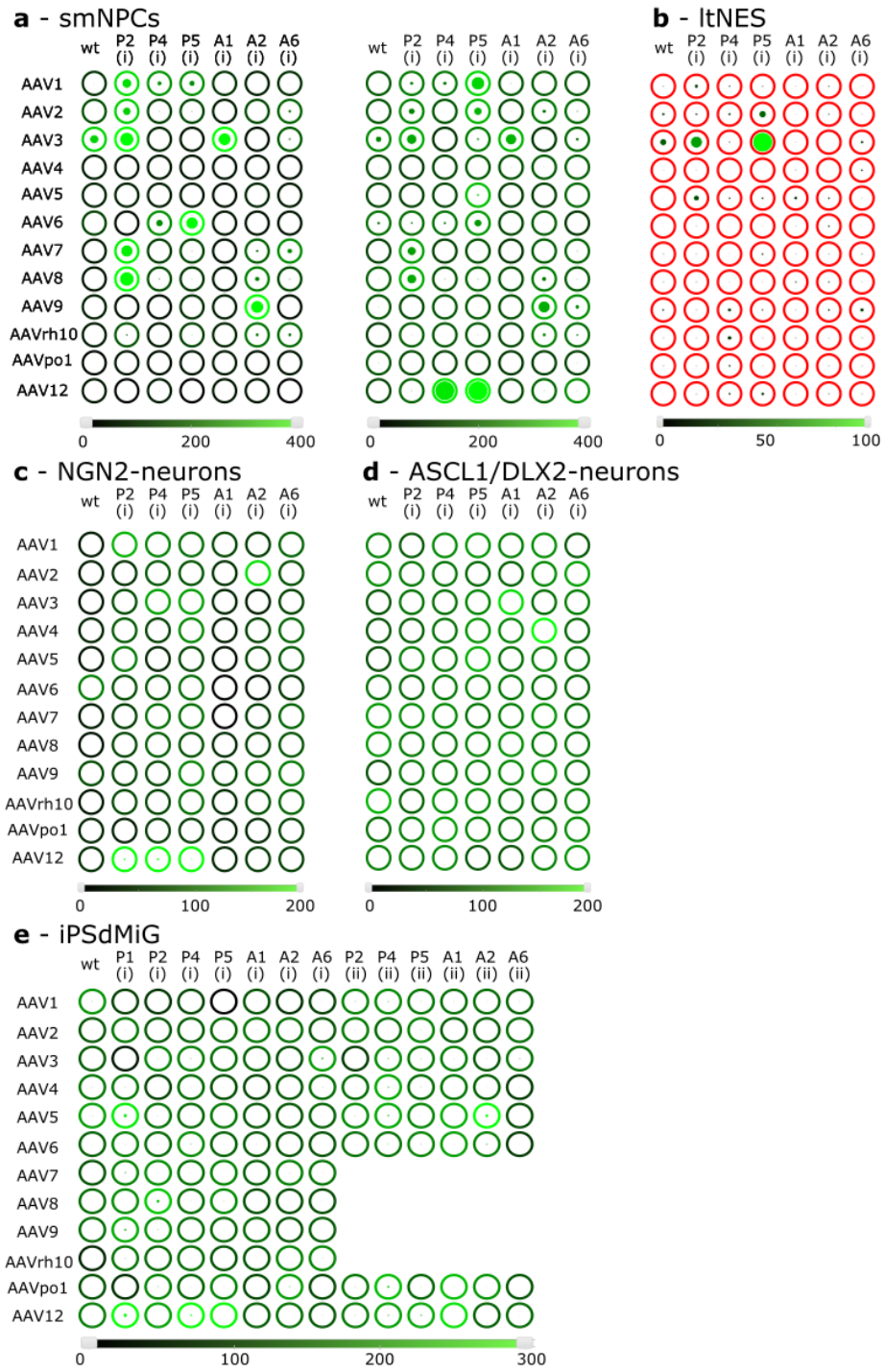
[a] *The peptide insertion sites are represented with flanking residues in the one-letter amino acids code. Depending on the serotype, more than one insertion site has been targeted.*

Supplementary Figures



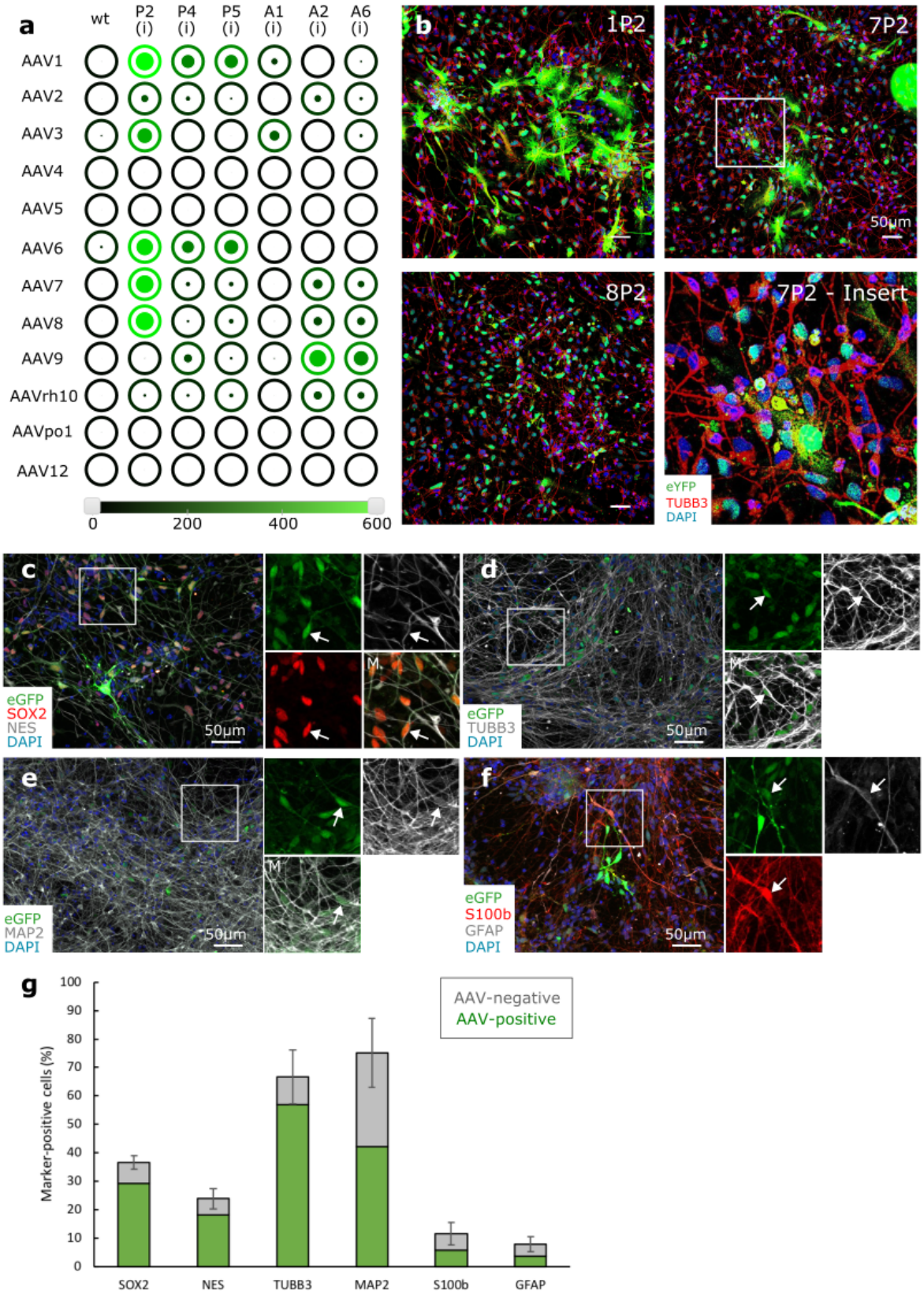
Supplementary Figure S1: Comparison of fluorescence measurements using a plate reader or automated image acquisition and analysis pipelines.

Raw data generated by different readout methods, *i.e.*, fluorescence measurements using automated image acquisition and analysis pipelines or a plate reader, after transduction of the AAV-YFP screening panel in iPSC-derived astrocytes.



Supplementary Figure S2: Overview on the transducability of human cell programming-derived CNS cell types.

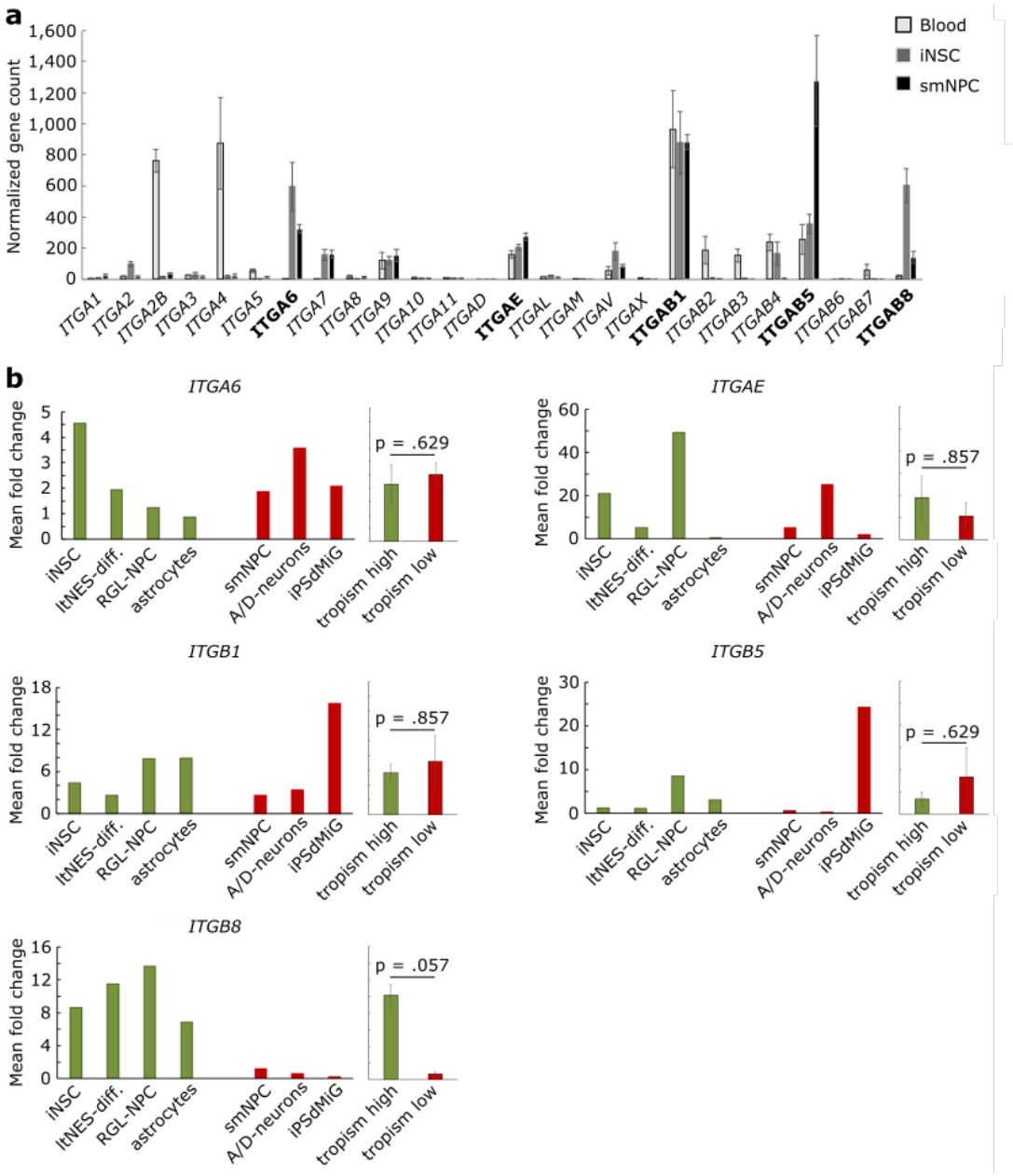
(a - e) Bubble charts representing the transduction efficiencies of all screened AAV-YFP variants in smNPCs (a), undifferentiated ltNES (b), forward programmed NGN2- (c) and ASCL1/DLX2-neurons (d) as well as iPSdMiG (e). Panels a, c, d and e are based on the quantification of fluorescence intensities according to imaging data, whereas panel b is based on plate reader measurements. Plate reader-derived data are identified by red outlines. wt = wild-type. Inserted peptides (P1, P2, P4, P5, A1, A2, A6) and insertion sites (i, ii) are described in Supplementary Tables S1 and S2, respectively.



Supplementary Figure S3: Characterization of cell type-specific transduction in ltNES-derived neuroglial differentiation cultures.

(a) Bubble chart representing transduction efficiencies (cycle filling; in %) and absolute expression levels (color scalebar; mean gray value of all cells in the analyzed images) of all screened AAV-YFP

variants in ltNES-derived neuroglial differentiation cultures after immunofluorescence analysis with an antibody to TUBB3. **(b)** Representative images illustrating the existence of TUBB3/YFP-double-positive cells in AAV screening plates, indicating the general capability of selected AAV variants to transduce neurons. **(c-f)** Representative pictures of ltNES-derived differentiation cultures after transduction with a pool of three GFP-encoding AAVs (AAV1P2, 7P2 and 8P2). Immunofluorescence stainings were performed for markers of NSCs (SOX2, NES; c), differentially mature neurons (TUBB3, MAP2; d, e) and glial cells (S100b, GFAP; f). Arrows within the magnified areas (*i.e.*, white rectangles in the source picture) point toward examples of GFP-positive cells, which are also immunopositive for the assessed markers. Close-ups denoted with 'M' are merged overlays of the provided single channel pictures. **(g)** Quantification of immunofluorescence stainings shown in figure panels c-f. N = 5-6 images per marker.



Supplementary Figure S4: Integrin expression in different human cell populations.

(a) Normalized gene counts of integrin family members in human blood cells, directly converted iNSCs and iPSC-derived smNPCs. RNA sequencing data used for this analysis have been published before.[20] Integrins printed in bold are the five family members with highest expression levels in iNSCs. Bar graphs represent means ± standard error with N = 3-9 independent samples per group. **(b)** qPCR-based expression profiling of the five integrins with highest gene counts in iNSCs across different human cell programming-derived cell types. Left panels in each graph depict the individual data of all cell types studied here, whilst the panels on the right depict the means of all well or poorly transduced types (tropism high or low, respectively). Wilcoxon signed rank tests were performed on the pooled datasets. Bar graphs depict mean fold changes ± standard error, if applicable, normalized to commercial human fetal brain (18-19 weeks of gestation; Agilent Technologies, Santa Clara, USA). A/D-neurons = ASCL1/DLX2-induced neurons.

Supplementary References

54. Michelfelder, S. *et al.* Successful expansion but not complete restriction of tropism of adeno-associated virus by in vivo biopanning of random virus display peptide libraries. *PLoS One* **4**, e5122 (2009).

55. Waterkamp, D.A., Muller, O.J., Ying, Y., Trepel, M. & Kleinschmidt, J.A. Isolation of targeted AAV2 vectors from novel virus display libraries. *J. Gene Med.* **8**, 1307-1319 (2006).