High throughput screening of novel AAV capsids identifies variants for transduction of adult NSCs within the subventricular zone

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The adult mammalian brain entails a reservoir of neural stem cells (NSCs) generating glial cells and neurons. However, NSCs become increasingly quiescent with age, which hampers their regenerative capacity. New means are therefore required to genetically modify adult NSCs for re-enabling endogenous brain repair. Recombinant adeno-associated viruses (AAVs) are ideal gene-therapy vectors due to an excellent safety profile and high transduction efficiency. We thus conducted a high-throughput screening of 177 intraventricularly injected barcoded AAV variants profiled by RNA sequencing. Quantification of barcoded AAV mRNAs identified two synthetic capsids, peptide-modified derivative of wild-type AAV9 (AAV9_A2) and peptide-modified derivative of wild-type AAV1 (AAV1_P5), both of which transduce active and quiescent NSCs. Further optimization of AAV1_P5 by judicious selection of the promoter and dose of injected viral genomes enabled labeling of 30%-60% of the NSC compartment, which was validated by fluorescence-activated cell sorting (FACS) analyses and single-cell RNA sequencing. Importantly, transduced NSCs readily produced neurons. The present study identifies AAV variants with a high regional tropism toward the ventricular-subventricular zone (v-SVZ) with high efficiency in targeting adult NSCs, thereby paving the way for preclinical testing of regenerative gene therapy.

INTRODUCTION
The adult brain has long been considered a tissue with no regenerative capacity, partly due to the absence of pluripotent cells. In the late 1990s, a reservoir of neural stem cells (NSCs) with the potential to generate glia and neuronal progeny was identified in the adult mammalian brain.1,2 The largest reservoir of NSCs in rodents is located along the walls of the lateral ventricles, the so-called ventricular-subventricular zone (v-SVZ). The potential of these NSCs to produce different glia and neuronal subtypes has been demonstrated by lineage-tracing studies.3-5 NSCs get activated to provide progeny for tissue homeostasis but also in the frame of a traumatic brain injury.6-11 However, the ability to activate NSCs highly declines with age,12 hampering repair of the brain. This fairly limited endogenous-regenerative capacity calls for new strategies to specifically target and genetically modify adult NSCs within the natural environment of the brain.

Many different viral and transgenic approaches have been developed in the past to manipulate adult NSCs and their progeny.13 For a long time, onco-retroviruses and lentiviruses that integrate their genomes into the host cellular chromatin were the tools of choice. However, limitations of integrating viruses,13 such as insertional mutagenesis,14,15 gradual silencing of the inserted transgene,16,17 and the fact that not all non-dividing cells are equally transduced in vivo,18 hamper their use for targeting of especially quiescent (q)NSCs within the v-SVZ. Over the last few years, the non-enveloped adeno-associated viral (AAV) vectors have taken center stage as a gene-delivery vehicle for human gene therapy with two gene therapeutic approaches that have gained regulatory approval for commercial use in patients: Glybera (uniQure) and Luxturna (Novartis), and with a large amount of AAV gene therapeutic strategies even in the CNS under clinical development, as reviewed in Hocquemiller et al.,20 Deverman et al.,21 Foust et al.,22 and Wang et al.23 AAVs are small virus particles, belonging to the dependoviruses within the parvoviridae family with a capsid diameter of ~22 nm that is sterically limiting its genome to ~4.7 kb.24 The original AAV genome consists of only two genes, the rep and cap gene, which are organized in three open reading frames. The cap gene determines

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the structure of the AAV capsid, whereas the rep gene is involved in several processes ranging from transcription initiation to packaging of the AAV genome. For vector production, these genes are commonly delivered in trans and thus can be easily modified.25–33 Over the last decades, hundreds of AAV isolates were identified in various species, with an interestingly high homology regarding their capsid protein amino acid sequences, e.g., up to 99% for the primate isolate AAV1 compared to the human isolate AAV6.34 Favorable safety profiles combined with the ability to mediate long-term transgene expression and to efficiently target many different human tissues are major assets that make AAVs a preferred technology.25,35–38

Nonetheless, specific targeting of NSCs in the v-SVZ has remained challenging to date. Whereas the most efficient wild-type (WT) serotype, AAV9, shows high transduction efficiency upon intravenous and intracranial injection, it mainly targets neurons and astrocytes, but not NSCs.32,39–41 Just recently, the power of structure-guided DNA shuffling was used to develop the newly engineered AAV variant SCh9. This new variant was able to target cells in the v-SVZ including NSCs.42 However, to date, the usefulness of AAV vectors for transduction of stem cells remains debated, mainly based on conflicting reports concerning their transduction efficiency as reviewed.43 The variable regions of the viral protein (VP), which is encoded by the cap gene, are involved in receptor binding and antibody recognition and thus modifications thereof can be used to guide targeting of specific cell types. Engineering of the AAV capsid for optimization of organ, region, or cell specificity can be achieved by methods such as random cap gene mutation, DNA family shuffling, or peptide display, combined with in vivo selection.44–50 Most recently, barcoding of double-stranded encapsidated DNA and next-generation sequencing (NGS) were shown to allow for high-throughput screening of AAV capsid libraries.51,52 Taking these advances as a platform, we apply here these barcoded AAV libraries by intracerebroventricular injection of the adult rodent brain in order to find an optimal candidate to transduce NSCs from the v-SVZ. By using a combination of NGS, immunohistochemistry (IHC), flow cytometry, and mathematical modeling, we validate transduction of the NSCs within the v-SVZ and their neurogenic lineage by the novel AAV capsid peptide-modified derivative of WT AAV1 (AAV1_P5).

RESULTS

To identify AAV capsids able to transduce NSCs in the v-SVZ with the highest transduction efficiency possible, we performed an NGS-based high-throughput screening of 177 different barcoded AAV capsid variants. These AAV variants comprise 12 AAV WTs, 94 newly generated peptide display mutants based on these WTs, and 71 chimeric capsids generated through DNA family shuffling. Among the synthetic capsids are 24 previously published benchmarks, with the remaining ones being generated as described in Materials and methods, in Table S4, and in greater detail in Weinmann et al.52 To assess the performance of individual AAVs, the capsid variants were uniquely barcoded with a 15-nucleotide (nt)-long random DNA sequence and packaged into an AAV vector expressing a cytomegalovirus (CMV) promoter-controlled eYFP (enhanced yellow fluorescent protein) that harbors the barcode in its 3’ untranslated region (UTR). A library comprising either 91 (library #1 from Weinmann et al.53) or 157 (library #3 from Weinmann et al.53) capsid variants was directly injected into the lateral ventricles of the adult mouse brain (1010 viral genomes [vgs] in 2 μl per mouse) (Figures 1A and S2A).

For AAV mRNA analysis, capsids were ranked within each sorted cell population by the relative expression of their cognate barcodes, normalized by their frequency within library #1 and library #3. Overall capsid rankings of the 71 capsids shared by both libraries revealed the same top candidates and correlated strongly (Spearman’s rank correlation ρ = 0.84, p < 0.01) (Figure 1D). Furthermore, we did not find a significant association between barcode guanine-cytosine (GC) content and frequency in either libraries S2L and S2M (Materials and methods), indicating that the...
results are not strongly influenced by GC bias. Further analysis revealed that two synthetic capsids, AAV1_P5 and AAV9_A2 (peptide-modified derivative of WT AAV9), stood out as the most efficient AAV capsid variants based on the ranking of their barcode enrichment (Figures 1D–I and S2B–S2K). Notably, both aNSC and qNSC were robustly transduced by these two AAV capsids (Figures 1F, 1G, 1I, and 1J). Besides, AAV1_P5 and AAV9_A2 transduced other v-SVZ cell types, such as TAPs (Figures S2B and S2G), neuroblasts (Figures S2C and S2H), astrocytes (Figures S2D and S2I), oligodendrocytes (Figures S2E and S2J), and ependymal cells (Figures S2F and S2K). These two lead candidates clearly outperformed the well-established AAV2 and AAV9 WT capsids across all v-SVZ-cell populations (Figures 1K and 1L), as well as the parent WT AAV1. Taken together, our study has successfully identified AAV capsids that were highly region specific for the v-SVZ, probably due to their inability to migrate out of this region as reported for the SCH9 variant. These candidates exhibited a higher efficiency in targeting both aNSC and qNSC than established WT AAV variants in the v-SVZ in vivo.

One potential application of gene therapy is to genetically modify freshly isolated cells and transplant them back to the donor. Hence, to identify the capsid with the fastest transduction rate of isolated NSCs, we assessed the expression dynamics of WT serotypes AAV2 and AAV9, respectively (AAV2_WT and AAV9_WT), AAV9_A2, and AAV1_P5 in NSCs in vitro. To detect viral transduction of targeted cells and their progeny, we took advantage of the recombination of pairs of loxP sites by the Cre recombinase (Cre/loxP) system and engineered the AAVs to express a CMV immediate enhancer/actin (CAG) promoter-controlled Cre recombinase fused to GFP (CAG_Cre::GFP). We decided to use the CAG promoter to assess performance of these capsids, since this promoter proved to outperform Cre::GFP. We decided to use the CAG promoter to assess performance of these capsids, since this promoter proved to outperform Cre::GFP. We decided to use the CAG promoter to assess performance of these capsids, since this promoter proved to outperform Cre::GFP. We decided to use the CAG promoter to assess performance of these capsids, since this promoter proved to outperform Cre::GFP. We decided to use the CAG promoter to assess performance of these capsids, since this promoter proved to outperform Cre::GFP. We decided to use the CAG promoter to assess performance of these capsids, since this promoter proved to outperform Cre::GFP. We decided to use the CAG promoter to assess performance of these capsids, since this promoter proved to outperform Cre::GFP. We decided to use the CAG promoter to assess performance of these capsids, since this promoter proved to outperform Cre::GFP.

Next, we investigated whether the newly identified AAV capsids AAV1_P5 and AAV9_A2 also target v-SVZ cells in vivo. To this end, we individually injected $10^7$ vgs of AAV9_A2, AAV1_P5, or the well-established AAV9_WT and AAV2_WT, all containing the CAG_Cre::GFP construct, into tdTomato-flox mice (Figure 2E). Notably, at 7 dpi, the tropism toward the v-SVZ highly differed between the tested capsids (Figures 2F and 2G). AAV2_WT and in particular AAV9_WT targeted many cells outside of the v-SVZ, especially in the medial and dorsal wall of the lateral ventricles, whereas the striatum was not targeted (Figures 2F and 2G and data not shown). In contrast to the WT capsids, AAV1_P5 and AAV9_A2 demonstrated a significantly higher tropism toward the v-SVZ (Figure 2I). AAV1_P5 showed the most unique tropism, with 98% of all tdTomato-labeled cells lying along the v-SVZ. In addition, transduction rates of overall cells also differed among the four capsids. AAV1_P5 and AAV9_A2 exhibited the fastest kinetics and most robust rate of transduction, with AAV1_P5 transducing the largest number of cells at 5 dpi as compared to the other capsids (Figures 2F). The overall number of transduced NSCs became similar at 7 dpi for all capsids except AAV2_WT (Figure 2F). Nevertheless, AAV9_WT mostly targeted cells lying outside of the ventricular wall that we clearly identified as neurons based on their morphology. This is in line with previous reports showing a high transduction efficiency of AAV9 for neuronal cells. By contrast, AAV1_P5 and AAV9_A2 exhibited a selective tropism for the v-SVZ, mainly targeting NSCs/TAPs (SOX2+/GFAP+/S100B−) as well as ependymal cells (SOX2+/S100B−; Figures 2H and 2J).

Along the wall of the v-SVZ, ependymal cells are organized in a so-called pinwheel architecture with NSCs in the center. Within these structures, ependymal cells outnumber NSCs, explaining why AAV1_P5 and AAV9_A2 transduce more ependymal cells overall. A recent report using single-cell transcriptomics and fate mapping of ependymal cells demonstrates their inability to generate progeny even after growth factor administration or brain injury. This ensures that progeny labeled with AAV1_P5 or AAV9_A2 stems from NSCs. However, manipulated ependymal cells communicate with neighboring NSCs and might indirectly change the progeny of these NSCs. To address this, strategies to de-target ependymal cells, by using a NSC-specific promoter or a microRNA (miRNA)-regulated viral vector, might be of use. The latter would require a screening for ependymal cell-specific miRNAs. Taken together, our data

Figure 2. Assessment of expression dynamics and v-SVZ targeting of the lead candidate AAV capsids

(A) Experimental outline to assess expression dynamics of AAV1_P5, AAV9_A2, and two WT capsids in vitro. (B) Representative images of NSCs in vitro transduced with different AAV capsids 7 days after injection (days post-injection [dpi]); scale bars, 20 μm. (C) Dynamics of tdTomato expression at different time points in primary-cultured NSCs. AAV9_WT, 3 dpi (11.9% ± 5.04%) versus AAV9_A2, 3 dpi (58.8% ± 8.24%) versus AAV1_P5, 3 dpi (44.4% ± 6.94%) (Kruskal-Wallis test followed by Dunn’s post hoc test). (D) Dynamics of GFP expression at different time points in primary-cultured NSCs. (C and D) Cultured NSCs were used up to passage 7; n = 3 cell cultures from 3 different mice. (E) Schematic illustration of the experimental outline to in vivo validate different AAV capsids. (F and G) IHC of the v-SVZ with markers to discriminate the different cell types after (F) AAV9_WT and (G) AAV1_P5 transduction (scale bars, 100 μm and 50 μm, respectively). (H) Markers for IHC used to discriminate the different cell types (NSCs left; Ep cells right; scale bars, 30 μm). (I) Proportion of tdTomato-labeled cells located in the v-SVZ among all tdTomato-positive cells in a 25-μm-thick coronal brain section. A high proportion indicates regional specificity for the v-SVZ. AAV9_WT (31.5% ± 5.9%) versus AAV9_A2 (3.84% ± 0.33%) versus AAV9_A2 (81.6% ± 10.1%) versus AAV1_P5 (98.9% ± 1.13%). (J) Dynamics of tdTomato expression at different time points in the full v-SVZ. Bars are partitioned by the mean proportion of cell types across mice. AAV2_WT, 5 dpi (0.06 ± 0.06) versus AAV1_P5, 5 dpi (4.67 ± 1.96) and AAV2_WT, 7 dpi (0.22 ± 0.11) versus AAV9_A2, 7 dpi (6.02 ± 0.71) versus AAV1_P5, 7 dpi (4.17 ± 1.20).
Figure 3. Single-cell RNA sequencing (scRNA-seq) reveals transduction of cells of the adult NSC lineage by AAV1_P5

(A) Experimental outline of labeling, isolation, and scRNA-seq of the adult NSC lineage using the AAV1_P5 capsid. (Top panel) Untransduced cells from the TiCY mouse line express neomycin resistance (NeoR). Cre recombinase (Cre)-mediated recombination induces the expression of eYFP and the loss of NeoR expression. AAV1_P5 loaded with Cre was delivered to the lateral ventricle of P91 TiCY mice. After 5 weeks, all labeled (eYFP+) cells from the v-SVZ and the rest of the brain (striatum, rostral migratory stream [RMS], and OB), as well as further unlabeled NSC lineage cells (GLAST+ from v-SVZ) were sorted and used for scRNA-seq. (B) 2D representation of the resulting 4,572 gene expression (mean z-score)
demonstrate a unique tropism and fast targeting of NSCs/TAPs and ependymal cells within the v-SVZ by AAV1_P5 and AAV9_A2.

To select the best candidate between AAV1_P5 and AAV9_A2 regarding NSC transduction efficiency, we performed FACS analysis of the v-SVZ and OB of injected mice. 2-month-old C57BL/6N mice were injected with $10^{10}$ vgs in 10 µL of either AAV1_P5 or AAV9_A2 capsids containing the eYFP reporter under the CMV promoter, as these were the capsids used for the barcoded libraries. 6 dpi, mice were sacrificed, and NSCs with their progeny from the v-SVZ and the OB neuroblasts were analyzed by FACS quantification (Figures S3C, S6A, and S6B). By determining the fraction of YFP+ cells among these cell types, we calculated the labeling efficiency of the different viruses. Our results show that AAV1_P5 has a higher labeling efficiency for NSC (11.19%) than the AAV9_A2 capsid (2.95%) (Figure S3D). This higher transduction efficiency could also be seen for qNSC, aNSC, TAPs, and negative binomials (NBs) from the SVZ (Figure S3D). This prompted us to proceed with the AAV1_P5 capsid for further experiments. Of note, the overall low number of detected YFP+ cells is due to the lower sensitivity of FACS analysis for YFP-expressing cells as compared to mCherry or tdTomato, as previously shown (Tlx-YFP (YFP expression under the Tlx [Nr2e1 nuclear receptor gene] promoter) versus Tlx-tdTOMATO in Baser et al.61). In addition, here, we directly measure the viral YFP as opposed to the measurement of tdTomato expression induced by AAV-Cre in Figure 1.

In order to test the ability of direct AAV1_P5-transduced NSCs to generate progeny, freshly isolated NSCs from tdTomato-irox mice were transduced with AAV1_P5 expressing Cre recombinase under the control of a CMV promoter (CMV_Cre). Thereafter, transduced cells were transplanted into the v-SVZ of C57BL/6N WT mice (Figure 3A, top). 37 dpi, we isolated cells from the v-SVZ and other brain regions as schematically depicted in Figure 3A. More precisely, we isolated labeled cells of the v-SVZ and the striatum, rostral migratory stream (RMS), and OB, here referred to as rest of the brain (RoB). To capture the remaining unlabeled cells of the NSC lineage in the v-SVZ, we also isolated GLAST+ v-SVZ cells (see Figures 3A and S4E–S4G for the proportion of cell populations). Two samples of two pooled mice each were subjected to scRNA-seq. Initial inspection of the resulting 4,572 single-cell transcriptomes revealed a segregation of proliferating cells as indicated by the expression of the proliferation marker protein Ki67 (MKI67) and canonical markers of G2/M and S phase (Figures S4H and S4I). After mitigating the effects of phase heterogeneity by regression, we obtained a continuous trajectory ranging from NSCs to late NBs (LN Bs)/immature neurons (glia markers, followed by increased expression of ribosomal genes and cell-cycle genes, and finally upregulation of neuron differentiation genes.6 Visualizing the expression of representative genes from Llorens-Bobadilla et al.6 recapitulated the same transcriptional progression in our dataset (Figure 3C). Only few eYFP+ off-target cells (sample #1: 9.7%; sample #2: 2.7%) were captured, consisting of mostly ependymal cells (Figure 3B). We found that cells isolated from RoB are located at the very end of this trajectory, as expected (Figure S4J).

Next, we sought to distinguish labeled (eYFP+ NeoR-negative [NeoR−]) cells from unlabeled (eYFP− NeoR-positive [NeoR+]) cells in our single-cell transcriptomes (Figures 3D and S4K). As expected (Figure 3A, top), eYFP-expressing cells mostly do not express NeoR, and vice versa, cells expressing NeoR mostly do not express eYFP. Only very few cells express both eYFP and NeoR (samples #1 and #2: 1.4% and 3.7%), possibly due to incomplete Cre-mediated excision. Transcripts of the viral Cre-recombinase, however, were rarely detected and mostly in early stages of the lineage but notably, also in very few cells at the end of the lineage, indicating an overall very low expression that prevents estimation of the dilution of viral transcripts along the lineage (Figure S4L). The floxed genes, eYFP and NeoR, exhibited higher expression than the Cre transcript. eYFP was more readily detected than NeoR, but ultimately, both genes suffered from the usual “dropout” in scRNA-seq, i.e., the failure to capture and/or detect transcripts.63 For a substantial fraction of cells, neither NeoR nor eYFP was detected. The fraction of such undistinguishable cells was larger in cells with fewer total detected transcripts such as qNSCs and LN Bs (Figures 3D and 3E). To overcome this issue and estimate AAV1_P5 transduction efficiency while accounting for single-cell transcriptomes. Most cells form one continuous trajectory from qNSCs to early NBs (ENBs; mostly from v-SVZ) and late NBs (LN Bs)/immature neurons (mostly from rest of brain). Few off-target cells including Ep cells and others (gray) were captured. (C) Mean relative gene expression of NSC lineage markers from Llorens-Bobadilla et al.6 and Ep cell markers from Shah et al.62 in each cluster of single cells. (D) Fraction of eYFP+ and NeoR+ single-cell transcriptomes by cell type (m, cells per group). (E) Total number of uniquely identified mRNA molecules (UMI count) per cell, separated by cell type. (F) Maximum likelihood estimate of the fraction of transduced cells, based on values in (C and D). LNB and ENB were sorted by eYFP+ only and act as a control with an expected transduction rate of 100%. (G) Expression of G2/M-phase marker genes across samples and cell types (clusters from B), distinguishing between eYFP+ and eYFP− cells. (H, left) MA plot of gene-expression differences between eYFP+ and eYFP− cells. (Right) log2 fold-change distribution for all genes (gray) and viral response genes (blue).
Figure 4. AAV1.5s targets qNSCs, and the choice of promoter and viral load determines the number of generated OB neurons.

(A) Schematic illustration of the experimental outline to test v-SVZ labeling at different time points. (B) IHC of the v-SVZ (scale bars, 50 μm) and OB (scale bars, 200 μm and 50 μm) in the high-labeled and low-labeled group 35 dpi of AAV. (C) Time dynamics of labeled cells. Each mouse is identified by one symbol. Due to the heterogeneity among individual mice, each mouse was assigned to one of two groups. The color of the symbols indicates to which group the respective mouse belongs. (D and E) Comparison of...

(legend continued on next page)
total transcript count per cell and the likely different expression strengths of eYFP and NeoR, we employed maximum likelihood estimation (Figure 3F and Materials and methods). LNBs (mostly from eYFP+ sorted RoB) and ependymal cells (GLAST+) were used as controls since we know that almost all of these cells are transduced. Overall, we estimated a high transduction efficiency ranging from 46% to 93% for the cell types of the v-SVZ lineage and estimated 92% to 100% transduction in cells used as controls.

Lastly, we assessed whether the transduced cells show transcriptomic differences arising from the viral transduction itself. Both eYFP+ and eYFP− aNSCs and TAPs showed high expression of commonly used G2/M-phase marker genes (Figure 3G), which suggests that transduction with AAV1_P5 does not affect proliferation. Differential gene-expression analysis between eYFP+ cells and eYFP− cells (Figure 3H) identified only 18 differentially expressed genes (Table S5), indicating that AAV1_P5 transduction affects their transcriptome only mildly. Furthermore, we did not find any concerted upregulation of viral response genes in this comparison or when comparing eYFP− cells to eYFP− NeoR+ cells (Figure S4M) or naive v-SVZ lineage cells from Kalamakis et al.12 (Figure S4N). In conclusion, we have combined scRNA-seq with lineage tracing using AAV1_P5 and found that transduction does not affect the expression of proliferation markers and overall only minimally affects the transcriptomic readout.

We next tested whether the transduction efficiency could be further optimized by the selection of promoter and number of injected vgs per mouse. To this end, we now packaged the CMV_Cre construct into the AAV1_P5 capsid and injected either 10^9 vgs per mouse as in Figures 2E–2J or an increased concentration of 10^10 vgs per mouse into tdTomato-flox mouse brains (Figure S5A). In all conditions, tdTomato-labeled cells were detected at high numbers in the v-SVZ, confirming specific v-SVZ targeting by the AAV1_P5 capsid (Figures S5B–S5D). Transduction of cells was over 60 times higher with the CMV_Cre construct (319.9 cells per section) (Figure S5D) than with CAG_Cre (4.8 cells per section) (Figure 2J) when injecting 10^9 vgs per mouse. By increasing the number of injected vgs from 10^9 to 10^10, we were able to further increase the number of labeled cells (Figure S5D) including NSCs/TAPs and ependymal cells (Figures S5F and S5G). However, the increased viral load also moderately increased the proportion of labeled cells located outside of the v-SVZ (Figure S5E).

We finally assessed the neurogenic function of transduced NSCs in vivo. To this end, we assessed the number of transduced NSCs in the v-SVZ and their neuronal progeny in the OB. 10^10 vgs/mouse of AAV1_P5 harboring the CMV_Cre construct were injected into the lateral ventricles of tdTomato-flox mice, and at 35 dpi, the number of labeled NSCs in the v-SVZ and OB interneurons was assessed (Figure 4A). We observed a high heterogeneity in the number of labeled cells probably due to differences in the injection site. It should be noted that the given coordinates are always relative to the average brain of a WT mouse. Therefore, smallest differences in the volume or orientation of the ventricle by slight inclination of the head within the stereotactic frame are potential sources of variability of the injection site. One set of animals exhibited a lower number of labeled cells in the SVZ and OB than the other (Figure 4B). Although a trend toward a reduced number of NSCs/TAPs at 35 dpi was detectable, NSCs still remained in the v-SVZ at this late time point (Figure 4C), suggesting that AAV1_P5 also targeted qNSCs.

To estimate the extent of targeting of the NSC compartment, we took advantage of our previously developed mathematical modeling framework for stem cell dynamics of v-SVZ.12 First, we extended our previously established model and calibrated it to the experimentally observed dynamics of TAPs and OB neurons (see Supplemental material [Mathematical modeling]). Instead of fitting the model to average cell counts across mice, we subdivided the data into two groups, with higher and lower labeling, as animals with high labeling in the v-SVZ exhibited a much higher number of labeled cells in the OB than animals with lower labeling (Figures 4D and 4E). Fitting of the model to the data, assuming that viral transduction does not affect cell kinetics and that the observed heterogeneity comes from different numbers of initially labeled NSCs and TAPs, the model indicates that approximately 37% of NSCs are labeled in the high-label group and 26% of NSCs in the other group (see Supplemental material). Moreover, the model indicates that in the low-labeled group, barely any TAP would be labeled at the initial time, whereas in the other group, a higher number of TAPs are initially labeled.

Finally, we employed our model to address whether the observed labeling would arise from direct targeting of qNSCs, aNSCs, or both. To this end, we simulated two scenarios where either only qNSCs or only aNSCs are targeted (Figure 4F). Our simulation indicates that the ratio of labeled qNSCs to aNSCs reaches the same value in both scenarios after approximately 4 days, due to transitions between the quiescent and active state. Altogether, comparison of a model fit to data is in line with the hypothesis that the number of initially transduced NSCs and TAPs differs between the two groups, that the cell dynamics exhibited by transduced cells are comparable to non-
transduced cells, and that AAV5_P5 can target up to 57% of the NSC pool.

To validate the model prediction of the label efficiency of the AAV1_P5 vector, we performed a FACS quantification experiment to directly assess the percentage of NSC and progeny that is labeled by the virus 8 dpi (Figure 4G). 5-month-old TiCY mice were injected with 10^7 vgs/mouse of AAV1_P5 harboring the CMV_Cre construct. FACS quantification analysis was performed as described previously (Figures S3C, S3D, S6A, and S6B), and the results showed 30.46% labeling efficiency for NSCs (Figure 4H; mean eYFP+-percentage of both samples), which is close to the 26% labeling efficiency predicted by the mathematical model (Supplemental material). The model also showed a good fit when applied to the FACS quantification experiment performed to choose the best candidate between AAV1_P5 and AAV9_A2. Moreover, the prediction of a high labeling group was validated by the observed labeling rate in the single-cell transcriptomics analysis (see Supplemental material).

**DISCUSSION**

Altogether, in this study, we have performed barcode-based *in vitro* and *in vivo* high-throughput screenings of two libraries of WT and engineered AAV capsids. Targeting of NSCs and especially qNSCs has only been demonstrated in the hippocampal dentate gyrus with the capsid AAV r3.45 and the African green monkey isolate AAV4 as well as recently in the v-SVZ using the newly engineered AAV variant SCH9.

Here, we have identified two lead candidates for efficient targeting of NSCs *ex vivo* and *in vivo*. We particularly characterized the novel capsid AAV1_P5 as highly region specific at targeting cells of the v-SVZ layer, including ependymal cells and NSCs, by IHC, FACS quantification, and scRNA-seq. We moreover show by IHC and scRNA-seq that NSCs targeted with AAV1_P5 were not noticeably affected in their migration and transcriptome and readily generated OB neurons. Furthermore, we demonstrate that the engineered capsid AAV1_P5 also labels qNSCs. We propose that qNSC labeling cannot only be achieved by direct targeting of qNSCs but also indirectly through transduction of aNSCs that would later give rise to qNSCs. Indeed, based on mathematical modeling of FACS counts, we predict that labeled cells redistribute between those states within less than 1 week. Therefore, the initial labeling proportion of qNSCs to aNSC is not crucial when stem cell dynamics are observed on a longer timescale.

AAV1_P5 clearly targets cells in the v-SVZ. Which molecular mechanism leads to efficient targeting of v-SVZ cells by AAV1_P5 is unknown. It was previously shown that the SCH9 variant binds heparan sulfate proteoglycans and galactose, both of which are present on NSCs in the v-SVZ. AAV1_P5 may act via a similar mechanism that would lead to a specific tropism for v-SVZ cells, but other molecular mechanisms are also possible. For instance, AAV1_P5 may be unable to migrate deeply into the ventricular wall, which would favor transduction of NSCs, or it may be that AAV1_P5 has properties that favor its survival or activity in the cerebrospinal fluid. To date, there are only a few cases where such mechanisms underlying altered viral properties of synthetic AAV capsids have been successfully elucidated. One example is the use of the zv/8 integrin as a receptor for a keratinocyte-specific AAV2. Another example was reported by several labs that have recently identified an interaction of AAV-PHP.B (a peptide-modified AAV9) with the glycosylphosphatidylinositol (GPI)-linked protein LY6A. Other than these, however, the receptors or interactions that are targeted by peptide-engineered or shuffled AAV variants typically remain enigmatic, as do the intracellular mechanisms underlying their novel features. Hence, the identification of the receptor for AAV1_P5 will be the subject of future studies. In this looming work, it will then also be interesting to study whether AAV1_P5 interacts with other host cell factors that have been identified over the years for critical for transduction with WT capsids, such as the widely used AAV receptor AAVR or intracellular elements such as the proteasome.

As a proof of concept, we show that AAV1_P5 labeling can be combined with scRNA-seq to characterize the transcriptomes of NSCs and their progeny from different brain regions. Surprisingly, the number of transduced off-target cells in this experiment (Figure 3B) was much lower than in our previous FACS-based experiments (Figure 2). A possible explanation is that the main source of off targets, ependymal cells, are hard to detect in scRNA-seq experiments: a previous study isolated 9,804 cells from the v-SVZ without marker preselection, and only 46 of them were ependymal cells. As a result, the low off-target percentages reported in Figure 3B should only be expected in scRNA-seq experiments. Our method of AAV1_P5 labeling, followed by scRNA-seq, paves the way for more complex lineage tracing experiments *in vivo*. Recent studies have used CRISPR-Cas9-induced genomic scars combined with scRNA-seq to enable clonal lineage tracing in embryonic development. AAVs could be used to induce genomic scars in specific cells at specific time points to enable clonal lineage tracing in adult tissues. We use our scRNA-seq data to further corroborate our assessment that NSCs are efficiently targeted and remain functional after transduction. Future studies using electrophysiology are required to assess whether the progeny generated by transduced NSCs is fully functional and able to integrate into the neuronal circuits of the OB.

Finally, we identified the combination of the CMV promoter and AAV1_P5 capsid as ideally suited to efficiently transduce NSCs in the v-SVZ. Our finding that the CMV outperforms the CAG promoter differs from previous studies overexpressing plasmids via *in utero* electroporation in the mouse brain.

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Future experiments will be needed to unravel and understand the mechanisms governing the properties of our candidates. Altogether, we believe that our study opens tantalizing avenues to genetically modify NSCs in their in vivo environment for the treatment of CNS disorders or brain tumors.

MATERIALS AND METHODS

Animals

In this work, the mouse lines C57BL/6N, TdTomato-flox, and TiCY were used. All mice were male and were age matched to 8 weeks, except for TiCY mice, which were 5 months old (for FACS quantification) and 3 months old (for scRNA-seq). Animals were housed in the animal facilities of the German Cancer Research Center (DKFZ) at a 12-h dark/light cycle with free access to food and water. All animal experiments were performed in accordance with the institutional guidelines of the DKFZ and were approved by the “Regierungspräsidium Karlsruhe” (Germany).

AAV vector production

The production of the AAV–barcoded library was done as previously published with some modifications: 159 distinct barcodes were inserted into the 3′ UTR of a YFP reporter under the control of a CMV promoter and encoded in a self-complementary AAV genome. Each of the barcodes was assigned to one AAV capsid from a total of 183 variants, which are described in more detail in the accompanying manuscript by Weinmann et al. Altogether, this library production included 12 AAV-WTs (AAV1 to AAV9, AVVrh.10, AAVpo.1, and AAV12), 94 peptide display mutants, and 71 capsid chimeras, which were created by DNA family shuffling. Isolation of synthetic capsids was performed in specific tissues or in our recent screens of AAV libraries in cultured cells, mouse liver tissue, or muscle. These synthetic capsids include a set of 12 AAV serotypes that were previously modified by insertion of over 20 different peptides in exposed capsid loops and that were recently characterized in established or primary cells. In the work of Weinmann et al., all barcoded capsids were pooled in different combinations to finally obtain three distinct libraries (#1, #2 [not used in the present work], and #3), with 91, 82, and 157 variants. Further details on library composition are found in the supplemental information of Weinmann et al. All capsid variants are detailed in Table S4. HEK293T cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Merck), 1% penicillin (pen)/streptomycin (strept) (Gibco; 10,000 U/mL pen and 10,000 µg/mL strept) and 1% L-glutamine (Gibco; 200 mM) at 37°C and 5% CO2. AAV vectors were produced by seeding HEK293T cells (4.5 × 10⁶ cells per dish) on 90–150, 15 cm tissue-culture dishes (Sigma). 2 days later, we performed a polyethylenimine (PEI; Polysciences) triple transfection by mixing 44.1 µg (3 × 14.7 µg) DNA of (1) a plasmid containing the recombinant AAV genome of interest, (2) an AAV helper plasmid carrying AAV rep and cap genes, and (3) a plasmid providing adenoviral helper functions for AAV production in a total volume of 790 µL H2O per culture dish. Separately, PEI (113.7 µg) and H2O were mixed in a total volume of 790 µL per dish, and NaCl (300 mM) was added 1:1 to both, PEI, or DNA solution. PEI was added dropwise to DNA and incubated for 10 min at room temperature, before finally adding the DNA/PEI mixture to the culture dishes. 3 days later, cells were scraped off in the media and collected by centrifugation (400 g, 15 min). The pellet was dissolved in 0.5 mL virus lysis solution (50 mM Tris HCl, Sigma), 2 mM MgCl2 (Sigma), and 150 mM NaCl (Thermo Fisher Scientific; pH 8.5) and was immediately frozen at −80°C. In total, 5 × freeze-thaw cycles were performed with the cell pellet prior to sonication for 1 min, 20 s. The cell lysate was treated with Benzonase (75 U/µL; Merck) for 1 h at 37°C, followed by a centrifugation step at 4,000 × g for 15 min. CaCl2 was added to a final concentration of 25 mM, and the solution was incubated for 1 h on ice, followed by centrifugation at 10,000 g for 15 min at 4°C. The supernatant was harvested, and a 1/4 vol of a 40% polyethylene glycol (PEG 8000; Biochemica) and 1.915 M NaCl (Thermo Fisher Scientific) solution was added prior to incubation for 3 h on ice. After centrifugation for 30 min at 2,500 × g and 4°C, the pellet was dissolved in resuspension buffer (50 mM HEPEs; Gibco), 0.15 M NaCl (Thermo Fisher Scientific), and 25 mM EDTA (Sigma) and was dissolved overnight. The solution was then centrifuged for 30 min at 2,500 × g and 4°C, and the supernatant was mixed with cesium chloride (CsCl; Sigma) to a final concentration of 0.55 g/mL. The refractive index was adjusted to 1.3710 using additional CsCl or buffer, as needed. Next, the vector particles were purified using CsCl gradient density centrifugation. Fractions with a refractive index of 1.3711 to 1.3766 comprising DNA-containing AAV particles were pooled and dialyzed against 1× PBS with a Slide-A-Lyzer dialysis cassette according to the manufacturer’s instructions (Thermo Fisher Scientific). Subsequently, the samples were concentrated by using an Amicon Ultra Centrifugal Filter (Millipore; 100,000 nominal molecular weight limit [NMWL], used to retain the viral particles) following the manufacturer’s instructions. The volume of the samples was reduced to 250–300 µL. AAV vectors were finally aliquoted and stored at −80°C.

The production of the AAV1_P5_YFP and AAV9_A2_YFP viruses for the FACS analysis experiment was done as described above, with the only modification that the vectors were purified using two iodoxanil gradients. Of note, the barcoded AAV library construct as well as the YFP construct were engineered as double-stranded AAV vectors. The constructs for CAG_Cre::GFP and CMV_Cre were engineered as a single-stranded AAV vector.

AAV vector titration

AAV vectors were titrated using quantitative real-time PCR as described in Senis et al. For the CAG_Cre::GFP construct, the primers and probe GFP_forward (fwd), GFP_reverse (rev), and GFP_probe were used, whereas Cre_fwd, Cre_rev, and Cre_probe were used for the CMV_Cre construct (Table S1). The qPCR was performed on a C1000 Touch Thermal Cycler equipped with a CFX384 Real-Time System (Bio-Rad) with the following conditions: initial melting for 10 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C and annealing/extension for 30 s at 55°C. A standard curve was considered as reliable when the coefficient of determination (R²) was greater than 0.985.

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Stereotactic injection
AAV vectors were stereotactically injected into the lateral ventricle by using the following coordinates calculated to bregma: anterior-posterior (AP) $-0.5$ mm, medio-lateral (ML) $-1.1$ mm, dorso-ventral (DV) $2.4$ mm. Mice received either $10^9$ or $10^{10}$ vgs/mouse in a total volume of $10 \mu$L. The AAV libraries were stereotactically injected into the lateral ventricle by using the following coordinates calculated to bregma: AP $-0.5$ mm, ML $-1.1$ mm, DV $2.4$ mm. Mice received $4 \times 10^{10}$ vgs/mouse in a total volume of $2 \mu$L. Ex vivo-manipulated cells ($7,000$ FACS events) were injected into two areas of the v-SVZ using the following coordinates calculated to bregma: AP $0.7$ mm, ML $1.6$ mm, DV $2$ mm and AP $0$ mm, ML $1.7$ mm, DV $2$ mm.

Cell isolation and in vitro cultivation
The lateral v-SVZ was micro-dissected as whole mount as previously described. Tissue of single mice was digested with trypsin and DNase according to the guidelines of the Neural Tissue Dissociation Kit (trypsin; Miltenyi Biotec) using a Gentle MACS Dissociator (Miltenyi Biotec). Cells were cultured and expanded for 8–12 days in neurobasal medium (Gibco) supplemented with B27 (Gibco), heparin (Sigma), glutamine (Gibco), pen/strep (Gibco), epidermal growth factor (EGF; PromoKine), and fibroblast growth factor (FGF; PeloBiotec), as reported in Walker and Kempermann.

In vitro transduction of cultured NSCs
For RNA-seq, NSCs were seeded in 48-well plates (Greiner Bio-One) and incubated overnight. AAV library #1 or library #3 (same libraries as in Weinmann et al.; multiplicity of infection [MOI]: $10,000$) was added to the media and remained for the duration of 7 days. For IHC, Labtek chambers (Thermo Fisher Scientific) were coated with Poly D-Lysine (PDL; Sigma) laminin (Sigma), and NSCs were seeded at a density of $2 \times 10^4$ cells per square centimeter overnight. AAVs were added (MOI: $10,000$) and remained in the media for 1, 3, 5, or 7 days.

Single-cell transcriptomic profiling by 10x chromium 3’ sequencing

Stereotactic injection, single-cell suspension preparation, and sorting
3-month-old TiCY mice were stereotactically injected into the lateral ventricle with $10^9$ vgs of the AAV1_P5_Cre capsid. After 5 weeks of chase time, the mice were sacrificed, and the SVZ, striatum, RMS, and OB were isolated. The latter three tissues were pooled as a single tube. From the RoB, only eYFP+ cells were sorted. The total number of sorted events for the 2 days of the experiment was $12,000$ for SVZ cells and $5,800$ for cells of the RoB. TiCY mice were pooled for each sorting day. All of the cells were sorted in a volume of $50 \mu$L of fetal calf serum (FCS) $10\%$ in PBS, from which $45 \mu$L was used for loading the Chromium Next GEM Chip G.

Library preparation, sequencing, and mapping
One library per each sorting day was prepared by following the manufacturer’s protocol (Chromium Next GEM Single Cell 3’ version [v.] 3.1) and sequenced on a NovaSeq 6K PE 100 S1.

In order to quantify eYFP and NeoR (NeoR/kanamycin resistance gene) expression, entries for these transgenes were manually added to the FASTA and Gene Transfer Format (GTF) files of the mouse reference genome mm10-3.0.0 provided by 10X Genomics. scRNA-seq reads were pseudoaligned and further processed with kallisto|bus-tools\textsuperscript{83,84} to generate a gene × barcode count matrix.

Computational analysis of scRNA-seq data
Cell barcodes with less than 1,500 unique molecular identifiers (UMIs) or more than 15% mitochondrial reads were filtered, and the remaining cells were further analyzed in Scumpy v.1.5.1.\textsuperscript{85} We used Scany to calculate G2/M- and S-phase scores for all cells, based on their expression of G2/M- and S-phase marker genes from Tirosh et al.\textsuperscript{86} These scores were then regressed out of the count data to reduce the influence of the cell cycle on clustering. The first 50 principal components of $3,324$ highly variable genes were used for 2D visualization with Uniform Manifold Approximation and Projection (UMAP; n_neighbors = 35) and cell clustering with the Leiden algorithm (resolution = $0.5$). Cell clusters were assigned to cell types based on the expression of NSC lineage marker genes previously described in Kalamakis et al.\textsuperscript{12} and Llorens-Bobadilla et al.\textsuperscript{6} and ependymal cell markers from Shah et al.\textsuperscript{58} (Figure 3C). To identify the location of cells from RoB, kernel density estimates of cell density in the 2D UMAP space were calculated for both samples. Since sample #1 contains more RoB cells, and sample #2 contains more v-SVZ cells, we subtracted both densities to highlight cells that most likely stem from RoB (orange cells in Figure S4H).

In order to estimate transduction efficiency from scRNA-seq data, we used the following model, based on the usual approach of modeling RNA-seq counts by the NB distribution:

For non-transduced cells, we assume that they express NeoR such that an expected fraction $\mu_R$ of all of their mRNA transcripts originates from this gene. For each individual cell $j$, the actual expression strength $q_j^R$ of the gene varies around this expectation according to a gamma distribution with mean $\mu_R$ and variance $\sigma_R^2$. The observed number of UMIs is then modeled as a Poisson variable:

$$k_j^R | q_j^R \sim \text{Pois}(s_j q_j^R),$$

where $s_j$ is the total UMI count for cell $j$, summed over all genes. Marginalizing out $q_j^R$:

$$k_j^R \sim \text{Pois}(s_j \mu_R)$$

https://www.codecogs.com/eqnsuite.php?latex=q_j^R \sim \text{Pois}(s_j \mu_R)$$

we find $k_j^R$ https://www.codecogs.com/eqnsuite.php?latex=k_j^R \sim \text{Pois}(s_j \mu_R)$ to follow a NB distribution with mean $s_j \mu_R$ https://www.codecogs.com/eqnsuite.php?latex=k_j^R \sim \text{Pois}(s_j \mu_R)$ and dispersion $\sigma_R^2$. As we are looking at a non-transduced cell, the UMI count $k_j^R$ https://www.codecogs.com/eqnsuite.php?latex=k_j^R \sim \text{Pois}(s_j \mu_R)$ is, of course, zero.
Similarly, we write $k_j^Y$ and $k_j^R$ for the corresponding quantities of eYFP, expressed by transduced cells. For a fully transduced cell $j$, we therefore have $k_j^Y = 0$ but $k_j^R > 0$ for the unequal distribution of eYFP+ and eYFP− cells. For transduced cells with incomplete or heterozygous Cre-mediated excision, we should see both genes expressed but will model the expression strength to be only one-half as strong.

The likelihood of observing UMI counts $\mathbb{S}$ can be expressed by the Poisson distribution.

$$L_j = p_{U \mid NB} \left( k_j^u ; \mu_U, \alpha_U \right) + p_R \delta(k_j^R) + p_{F \mid NB} \left( k_j^F ; \mu_F, \alpha_F \right)$$

$$= p_{U \mid NB} \left( k_j^u ; \mu_U, \alpha_U \right) + p_R \delta(k_j^R) + p_{F \mid NB} \left( k_j^F ; \mu_F, \alpha_F \right)$$

where $p_{U \mid NB} = \frac{1}{\alpha_U + 1} e^{-\frac{\mu_U}{\alpha_U + 1}}$ and $p_R = \frac{\alpha_R}{\alpha_U + 1}$. The likelihood is expressed as a Poisson distribution for UMI counts $k_j^u$, and we use the Dirac delta function to model the presence of transduced cells $k_j^R > 0$. The parameters $\mu_U$ and $\alpha_U$ are the mean and dispersion, respectively, for the UMI counts within each sample, and $\alpha_R$ is the indicator function, i.e., $\alpha_R = 1$ if the cell is transduced, and $\alpha_R = 0$ if the cell is not transduced. The shared clusters as covariate. Genes with the Gene Ontology (GO) term “GO:0009615—response to virus” were highlighted.

**FACS**

Generation of single-cell suspension was performed as described in Llorens-Bobadilla et al. Cells were stained with the following antibodies: O4-allophycocyanin (APC) and O4-APC-Vio770 (Miltenyi; diluted 1:50), Ter119-APC-Cy7 (BioLegend; 1:100), CD45-APC-Cy7 (Becton Dickinson (BD); 1:200), GLAST (ACSA-1)-phycoerythrin (PE; Miltenyi: 1:20), CD9-eFluor450 (eBioscience; 1:300), Alex-647::EGF (Life Technologies; 1:100), CD9-PE-Cy7 (eBioscience; 1:75), CD24-PE-Cy7 (eBioscience; 1:75), and Sytox Blue (Life Technologies; 1:200). FACS analysis of AAV-injected mice was performed by two methods. The first method consisted of

**FACS analysis of AAV-injected mice**

FACS analysis for testing the transduction efficiency of the candidate viruses was performed by two methods. The first method consisted of...
infecting 5-month-old TiCY mice with the AAV1_P5_Cre virus, and after 8 days, SVZ and OB cells were FACS analyzed (Figures 4G and 4H). In the second method, we injected 2-month-old C57BL/6N mice with AAV1_P5_YFP and AAV9_A2_YFP viruses and analyzed them after 6 days (Figures S3C and S3D).

For FACS quantification of AAV-injected NSC/progeny, cells were sorted with the following antibodies: O4-APC-Vio770 (Miltenyi; diluted 1:100), CD45-APC-Cy7 (BD; 1:200), Ter119-APC-Cy7 (BioLegend; 1:100), GLAST (ACSA-1)-PE (Miltenyi; 1:50), Prominin1-APC (eBioscience; 1:75), PSA-NCAM-PE-Vio770 (Miltenyi; 1:50), Texas-Red::EGF (Life Technologies; 1:75).

**Ex vivo treatment of NSCs**

FACS NSCs were transduced with AAV (MOI: 10,000) and incubated on ice for 2–3 h. Cells were centrifuged for 15 min at 300 × g, 4°C, and were washed twice with PBS. The pellet was dissolved in 4 μL PBS.

**RNA isolation and cDNA synthesis**

RNA was isolated by using the PicoPure RNA Isolation Kit (Thermo Fisher Scientific). For RNA isolation of in vitro-transduced cells, 1,500 cultured NSCs per set were lysed in 100 μL extraction buffer. For isolation of FACS in vivo-transduced cells, batches of 500 cells or less were generated and were lysed in 100 μL extraction buffer. Up to 6 batches (2,500 cells) were obtained per set, depending on the cell type (Tables S2 and S3). The cell-containing extraction buffer was incubated for 30 min at 42°C, and the lysate was frozen at −80°C to increase the amount of isolated RNA. The cell lysate was mixed 1:1 with 70% ethanol, and RNA was extracted according to the guidelines of the PicoPure RNA Isolation Kit (Thermo Fisher Scientific). RNA was dissolved in 11 μL nuclease-free H2O. The cDNA synthesis was performed as described in Picelli et al. by using locked nucleic acid-template switch oligo (TSO) (Table S1) and by using either 14 cycles for in vitro-cultured NSCs or 15 cycles (>300 cells per batch) or 16 cycles (<300 cells per batch) for FACS in vivo-transduced cells for the cDNA enrichment step. After purification using AMPure XP beads (Beckman Coulter), cDNA was dissolved in 10 μL H2O.

**Barcode amplification PCR and NGS library preparation**

Barcodes were PCR amplified by using 10 ng cDNA as input material. Therefore, the PCR primers barcode_forward (Bar_fwd) and barcode_reverse (Bar_rev) that bind up and downstream of the 15-bp-long barcodes within the according cDNA were engineered, and the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used according to its manual in combination with 10 mM dNTPs (Thermo Fisher Scientific) (Table S1). The PCR was performed on a T100 Thermal Cycler (Bio-Rad) with the following conditions: initiation for 30 s at 98°C, followed by 35 cycles of denaturation for 10 s at 98°C, annealing/extension for 20 s at 72°C, and a final step for 5 min at 72°C. The result was a 113-bp-long PCR amplicon that includes the barcode with its 15-bp-long random DNA sequence. The PCR amplicon was AMPure XP bead purified (Beckman Coulter) with a bead/sample ratio of 0.8:1 in the first round and 1:1 in the second round. After this step, the samples were enriched for the barcode containing amplicon, and of course, the samples potentially contained the range of up to 157 different AAV barcodes, which were initially used. Next, 10 ng or 15 ng (library #1 or #3, respectively) of PCR amplicon was used for NGS library preparation with the NEBNext Chromatin Immunoprecipitation (ChIP)-Seq Library Prep Reagent Set for Illumina (NEB) for samples from library #1 and the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB) for samples from library #3. Multiplexed libraries were generated by following the manual and by using the NEBNext Multiplex Oligos for Illumina (NEB). All multiplexed samples for library #1 and library #3 are listed in Tables S2 and S3. For sequencing, up to 50% of PhiX were spiked in to increase the complexity of the library.

**Immunocytchemistry**

Cells were washed 3 × 5 min in PBS at room temperature, followed by a 30 min blocking step in PBS containing 0.3% horse serum [Millipore] and 0.3% Triton X-100 [Sigma]) at room temperature. Subsequently, the cells were incubated overnight in PBS containing primary antibodies at 4°C. Cells were washed in PBS for 3 × 5 min at room temperature and were incubated with secondary antibodies in PBS for 1 h in the dark at room temperature. Afterward, cells were washed 3 × 5 min in PBS and were mounted with Fluoromount G (eBioscience). The following antibodies were used: chicken anti-GFP (Aves; 1:1,000) and goat anti-mCherry (SICGEN; 1:1,000). Nuclei were counterstained with Hoechst 33342 (BioTrend; 1:3,000).

**Tissue preparation**

Animals were sacrificed by using an overdose of ketamine (120 mg/kg)/xylazine (20 mg/kg) and were subsequently transcardially perfused with ice-cold 20 mL 1× Hank’s balanced salt solution (HBSS; Gibco) and 10 mL of 4% paraformaldehyde (Carl Roth). The brains were dissected and postfixed in 4% paraformaldehyde overnight at 4°C. A Leica VT1200 Vibratome was used to cut the tissue in 50 μm (v-SVZ)- or 70 μm (OB)-thick coronal sections. From each mouse, three to six identical brain sections every 100 μm (v-SVZ) or 140 μm (OB) along the coronal axis were used for staining. Brain sections for staining the v-SVZ were harvested from 0.5 to 1.1 mm anterior to the bregma.

**IHC**

Brain sections were washed 4 × 10 min in Tris-buffered saline (TBS) at room temperature, followed by a 1-h blocking step in TBS (TBS with 0.3% horse serum [Millipore] and 0.3% Triton X-100 [Sigma]) at room temperature. The tissue was transferred to 0.5 mL Safe Lock Reaction Tubes containing 200 μL TBS+ including primary antibodies. Samples were incubated for 24–48 h at 4°C. Tissue samples were washed 4 × 10 min in TBS at room temperature, followed by a 30-min blocking step in TBS+ at room temperature. Brain sections were transferred to 0.5 mL Safe Lock Reaction Tubes containing 200 μL TBS+ including secondary antibodies. Samples were incubated in the dark for 2 h at room temperature. Subsequently, brain slices were washed 4 × 10 min in TBS at room temperature and were mounted on glass slides with Fluoromount G (eBioscience). The following antibodies were used: mouse anti-Sox2 (Abcam; 1:100),...
guinea pig anti-DCX (Merck; 1:400), rabbit anti-S100B (Abcam; 1:100), goat anti-mCherry (SICGEN; 1:1,000), and chicken anti-GFAP (GeneTex; 1:500). Nuclei were counterstained with Hoechst 33342 (BioTrend; 1:3,000).

**Microscopy and cell quantification**

All images were acquired with a Leica TCS SP5 Acousto-Optical Beam Splitter (AOBS) confocal microscope equipped with a UV diode 405 nm laser, an argon multiline (458–514 nm) laser, a helium-neon 561 nm laser, and a helium-neon 633 nm laser. Images were acquired as multichannel confocal stacks (z plane distance 3 μm) in 8-bit format by using a 20× or 40× oil-immersion objective at a resolution of 1,024 × 1,024 and 200 Hz. For quantification of the v-SVZ and total brain sections, tile scans of the whole ventricle or the whole coronal brain section were acquired with a total z stack size of 25 μm. To quantify the OB, tile scans of the whole OB covering the tissue thickness were acquired. For stained cells from in vitro culture, 4–9 fields of view were imaged. For representative images (2,048 × 2,048 resolution, 100 Hz), the maximum intensity of a variable number of z planes was stacked to generate the final z projections. Representative images were cropped, transformed to RGB color format, and assembled into figures with Inkscape (inkscape.org). For cell quantification, ImageJ (NIH) was used including the plug-in cell counter to navigate through the z stacks. To quantify cells in the OB, the volume of the OB was calculated by multiplying the entire area of every OB section (including the glomerular layer [GLL]) with the entire z stack size. Then we converted cubed micrometers to cubed millimeters. Finally, cell counts were given as cells/cubed millimeters OB. To elucidate the labeling efficiency of the different AAV variants in the total v-SVZ (medial, dorsal, and lateral wall of the lateral ventricle), the cells were counted on 25 μm-thick coronal sections and are given as cells per 25 μm section. Mainly NSCs located in the lateral wall of the ventricle generate OB neurons during homeostasis. Since a particular area of the lateral v-SVZ serves cells to a particular volume of the OB, cell numbers were counted for the mathematical modeling of the lateral v-SVZ only. The length of the lateral ventricular wall was measured in a coronal section and multiplied with the z stack size (25 μm) to estimate the area of the lateral v-SVZ. Afterward, cells in the lateral v-SVZ were counted and normalized to the lateral v-SVZ area. Data are given as cells per cubed millimeters.

**NGS screening of barcoded AAV capsid variants—computational analysis**

NGS samples were sequenced and demultiplexed by the DKFZ Genomics and Proteomics Core Facility using bcl2fastq 2.19.0.316. This resulted in two (paired-end) FASTQ files per sample. Each FASTQ consists of reads resulting from the targeted barcode amplification and up to 50% PhiX DNA that was spiked in to increase library complexity.

Each AAV variant is associated with a unique 15-mer barcode sequence. To quantify the most successful AAV, we simply counted how often each barcode occurred in each FASTQ file, bearing in mind the following pitfalls:

1. Barcode sequences might occur outside of the amplicon by chance, e.g., in the PhiX genome.
2. Barcodes might have sequencing errors.
3. Barcodes occur on the forward and reverse strand.

To circumvent issues (1) and (2), we opted for a strategy where we only count barcodes matching the expected amplicon structure. This was achieved with the following regex (regular expression; defines a text search pattern): (? \[NGCAT\]\{15\}[NGCAT\]{15}\(=\ [CAGG\[NGCAT\]\{15\})]. Variable 15-mers [NGCAT]\{15\} are only counted if they are flanked by the expected regions TGCTC and CAGGG. Furthermore, we enforce a minimum of 33 upstream nt and 15 downstream nt, in addition to the flanking regions, to only count 15-mers at the expected position. 15-mers matching this regex were extracted and counted with the standard GNU command-line tools grep, sort, and uniq. 15-mers sequenced from the reverse strand were counted with an equivalent reverse complement regex and added to the forward counts.

**Assigning barcodes to AAV capsids**

Raw 15-mer counts were further processed in R. Most observed 15-mers matched a known barcode exactly (library #1: 74%; library #3: 87%), which allowed us to assign them to a unique AAV variant. The remaining 15-mer counts were added to the counts of the closest known barcode, allowing for a maximum of two mismatches.

**Normalization**

Each sequenced sample corresponds to one tube with up to 500 FACS cells. To downweigh samples with lower cell numbers, barcode counts were scaled by the respective number of FACS events (usually 500; Table S2). Barcode counts of the same cell type and biological replicate (termed “sets”) were then summed. The AAV libraries used for transduction contain slightly unequal proportions of AAV variants, which means that some AAV variants may have an advantage due to increased starting concentration. To remedy this problem, barcode counts were further scaled by their abundance in the transduction library (as determined by Weinmann et al.23) (Table S6), so that barcode counts corresponding to more frequent AAV capsids were decreased and vice versa.

To account for sequencing depth of the individual samples, normalized barcode counts were divided by the total number of valid barcodes in that sample, yielding normalized barcode proportions. A potential source of bias is that ampiclons with different barcodes may have different RT-PCR efficiencies. A previous study 49 on ten barcoded AAV variants found no such bias, but nonetheless, we evaluated one possible source of bias, barcode GC-content, in our own data. We found no significant association between barcode GC-
content and mean barcode proportion across all samples in either library (Figures S2L and S2M).

**Identification of candidate AAVs with high transduction efficiency**

To identify the most promising AAV variants, AAVs were ranked by the mean normalized barcode proportion within and across cell types (Figures 1D–I). AAV1_P5 and AAV9_A2 performed consistently well across replicates of both experiments and were selected for further validation.

**Mathematical modeling**

A detailed description on how the mathematical modeling was developed is given in Supplemental material.

**Statistics**

Statistical analyses were performed with R v.4.0.2 using one-way ANOVA followed by Tukey’s honest significant difference (HSD) post hoc test unless otherwise noted. Tukey’s HSD p values were corrected for multiple testing with the Benjamini-Hochberg procedure. The homogeneity of variance assumption of ANOVA was assessed using Levene’s test, and the normality assumption was assessed with the Shapiro-Wilk normality test. The respective p values are indicated in the figure legends. Figures were plotted with the R package ggplot2 and SigmaPlot 12.5.

**Data and code availability**

All sequencing data are available at the NCBI Gene Expression Omnibus (GEO) under GEO: GSE145172.

All scripts used in the analysis are available at https://github.com/LKremer/AAV-screening.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.07.001.

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**AUTHOR CONTRIBUTIONS**

S.D. was involved in project and experimental design and performed experiments including in vitro and in vivo screens, ex vivo NSC transplantation, and in vitro and in vivo validations. L.P.M.K. was responsible for the bioinformatics analysis of all in vitro and in vivo screens and sequencing experiments. S.K. and S.C. conducted the single-cell RNA sequencing experiment. S.C. conducted the FACS quantification of cells transduced with lead candidates. T.S. was responsible for the mathematical modeling of the in vivo data. J.W. provided the two AAV capsid libraries and contributed to experimental design. H.A. and A.L. helped in producing AAV vectors. A.M.-C. contributed to the development of the mathematical model, interpretation of data, and revision of the manuscript. D.G., S.A., A.M.-C., and A.M.-V. supervised the project and wrote the manuscript. A.M.-V. designed and coordinated the study. All authors have read and approved the final version of the manuscript.

**DECLARATION OF INTERESTS**

D.G. is a co-founder and shareholder of AaviGen GmbH. All other authors declare no competing interests.

**REFERENCES**

1. Lim, D.A., and Alvarez-Buylla, A. (2016). The Adult Ventricular-Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. Cold Spring Harb. Perspect. Biol. 8, a018820.
2. Ming, G.L., and Song, H. (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70, 687–702.
3. Merkle, F.T., Mirzadeh, Z., and Alvarez-Buylla, A. (2007). Mosaic organization of neural stem cells in the adult brain. Science 317, 381–384.
4. Fuentealba, L.C., Rompani, S.B., Obernert, K., Romero, R., Cepko, C.L., and Alvarez-Buylla, A. (2015). Embryonic Origin of Postnatal Neural Stem Cells. Cell 161, 1644–1655.
5. Merkle, F.T., Fuentealba, L.C., Sanders, T.A., Magno, L., Kessaris, N., and Alvarez-Buylla, A. (2014). Adult neural stem cells in distinct microenvironments generate previously unknown interneuron types. Nat. Neurosci. 17, 207–214.
6. Llorens-Bobadilla, E., Zhao, S., Baser, A., Saiz-Castro, G., Zwaal, K., and Martin-Villalba, A. (2015). Single-Cell Transcriptomics Reveals a Population of Dormant Neural Stem Cells that Become Activated upon Brain Injury. Cell Stem Cell 17, 329–340.
7. Arvidsson, A., Collin, T., Kirkik, D., Kokaia, Z., and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat. Med. 8, 963–970.
8. Parent, J.M., Vexler, Z.S., Gong, C., Derugin, N., and Ferriero, D.M. (2002). Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Ann. Neurol. 52, 802–813.
9. Thored, P., Arvidsson, A., Cacci, E., Ahlenius, H., Kallur, T., Darsalia, V., Ek达尔, C.T., Kokaia, Z., and Lindvall, O. (2006). Persistent production of neurons from adult brain stem cells during recovery after stroke. Stem Cells 24, 739–747.
10. Hou, S.W., Wang, Y.Q., Xu, M., Shen, D.-H., Wang, J.-J., Huang, F., Yu, Z., and Sun, F.-Y. (2008). Functional integration of newly generated neurons into striatum after cerebral ischemia in the adult rat brain. Stroke 39, 2837–2844.
11. Liu, F., You, Y., Li, X., Ma, T., Nie, Y., Wei, B., Li, T., Lin, H., and Yang, Z. (2009). Brain injury does not alter the intrinsic differentiation potential of adult neuroblasts. J. Neurosci. 29, 5075–5087.
12. Kalamakis, G., Brüne, D., Ravichandran, S., Bolz, I., Fan, W., Ziebell, F., Stiehl, T., Catalá-Martinez, F., Kupke, J., Zhao, S., et al. (2019). Quiescence Modulates Stem Cell Maintenance and Regenerative Capacity in the Aging Brain. Cell 176, 1407–1419.e14.
13. Enikolopov, G., Overstreet-Wadsche, L., and Ge, S. (2015). Viral and transgenic reporters and genetic analysis of adult neurogenesis. Cold Spring Harb. Perspect. Biol. 7, a018804.
16. Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., Samulski, R.J., and Muzyczka, N. (2014). AAV-mediated gene therapy for research
17. Xia, X., Zhang, Y., Zieth, C.R., and Zhang, S.C. (2007). Transgenes delivered by len-
18. He, J., Yang, Q., and Chang, L.-J. (2005). Dynamic DNA methylation and histone
19. Park, F., Ohashi, K., Chiu, W., Naldini, L., and Kay, M.A. (2000). Ef
20. Thomas, C.E., Ehrhardt, A., and Kay, M.A. (2003). Progress and problems with the
21. Deverman, B.E., Ravina, B.M., Bankiewicz, K.S., Paul, S.M., and Sah, D.W.Y. (2018).
22. Wang, D., Tai, P.W.L., and Gao, G. (2019). Adeno-associated virus vector as a plat-
23. Mendelson, E., Trempe, J.P., and Carter, B.J. (1986). Identi
24. Rose, J.A., Berns, K.I., Hoggan, M.D., and Koczot, F.J. (1969). Evidence for a single-
25. Samulski, R.J., and Muzyczka, N. (2014). AAV-mediated gene therapy for research
26. Janik, J.E., Huston, M.M., and Rose, J.A. (1984). Adeno-associated virus proteins:
27. Janik, J.E., Huston, M.M., and Rose, J.A. (1984). Adeno-associated virus proteins: origin of the capsid components. J. Virol. 52, 591–597.
28. Mendelson, E., Trempe, J.P., and Carter, B.J. (1986). Identification of the trans-acting Rep proteins of adeno-associated virus by antibodies to a synthetic oligopeptide. J. Virol. 60, 823–832.
29. Becerra, S.P., Koczot, F., Fabsch, P., and Rose, J.A. (1988). Synthesis of adeno-asso-
30. Sonntag, F., Schmidt, K., and Kleinschmidt, J.A. (2010). A viral assembly factor pro-
31. Johnson, F.B., Ozer, H.L., and Hoggan, M.D. (1971). Structural proteins of adeno-
32. Van Vliet, K.M., Blouin, V., Brummet, N., Aghandje-McKenna, M., and Snyder, R.O. (2008). The role of the adeno-associated virus capsid in gene transfer. Methods Mol. Biol. 437, 51–91.
33. Li, C., and Samulski, R.J. (2020). Engineering adeno-associated virus vectors for gene therapy. Nat. Rev. Genet. 21, 255–272.
34. Daya, S., and Berns, K.I. (2008). Gene therapy using adeno-associated virus vectors. Clin. Microbiol. Rev. 21, 583–593.
35. Xiao, X., Li, J., and Samulski, R.J. (1996). Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J. Virol. 70, 8098–8108.
36. Naso, M.F., Tomkowicz, B., Perry, W.L., 3rd, and Strohl, W.R. (2017). Adeno-
37. Lykken, E.A., Shyg, C., Edwards, R.I., Rozenberg, A., and Gray, S.J. (2018). Recent progress and considerations for AAV gene therapies targeting the central nervous system. J. Neurodev. Disord. 10, 16.
38. Colella, P., Ronzitti, G., and Mingozi, F. (2017). Emerging Issues in AAV-Mediated In Vivo Gene Therapy, Mol. Ther. Methods Clin. Dev. 8, 87–104.
39. Cearley, C.N., and Wolfe, J.H. (2006). Transduction characteristics of adeno-associ-
40. Gray, S.J., Nagabushan Kalburgi, S., McCown, T.J., and Jude Samulski, R. (2013). Global CNS gene delivery and evasion of anti-AAV-neutralizing antibodies by intra-
41. Gray, S.J., Matagne, V., Bachaboina, L., Yadav, S., Ojeda, S.R., and Samulski, R.J. (2011). Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. Mol. Ther. 19, 1058–1069.
42. Ojala, D.S., Sun, S., Santiago-Ortiz, J.L., Shapiro, M.G., Romero, P.A., and Schaffer, D.V. (2018). In Vivo Selection of a Computationally Designed SCHEMA AAV LVYields a Novel Variant for Infection of Adult Neural Stem Cells in the SVZ. Mol. Ther. 26, 304–319.
43. Brown, N., Song, L., Kollu, N.R., and Hirsch, M.L. (2017). Adeno-Associated Virus Vectors and Stem Cells: Friends or Foes? Hum. Gene Ther. 28, 450–463.
44. Kienle, E., Senis, E., Börner, K., Niöpek, D., Wiedtke, E., Grosse, S., and Grimm, D. (2012). Engineering and evolution of synthetic adeno-associated virus (AAV) gene therapy vectors via DNA family shuffling. J. Vis. Exp. 3819.
45. Svistavasta, A. (2016). Adeno-Associated Virus: The Naturally Occurring Virus Versus the Recombinant Vector. Hum. Gene Ther. 27, 1–6.
46. Körbelin, J., and Trepel, M. (2017). How to Successfully Screen Random Adeno-Associated Virus Display Peptide Libraries In Vivo. Hum. Gene Ther. Methods 28, 109–123.
47. Bünning, H., and Svistavasta, A. (2019). Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors. Mol. Ther. Methods Clin. Dev. 12, 248–265.
48. Deverman, B.E., Pravdo, P.L., Simpson, B.P., Kumar, S.R., Chan, K.Y., Banerjee, A., Wu, W.-L., Yang, B., Huber, N., Pasca, S.P., and Gradinaru, V. (2016). Cre-dependent sequencing of Adeno-Associated Viruses. ACS Synth. Biol. 5, 194–206.
49. Stromberg, N., and Zolotukhin, S. (2015). E Pluribus Unum: 50 Years of Research, Millions of Viruses, and One Goal—Tailored Acceleration of AAV Evolution. Mol. Ther. 23, 1819–1831.
50. Adachi, K., Enoki, T., Kawano, Y., Veraz, M., and Nakai, H. (2014). Drawing a high-
51. Marsic, D., Méndez-Gómez, H.R., and Zolotukhin, S. (2015). High-accuracy bio-
52. Weinmann, J., Koller, T., and Sippel, J. (2016). In Vivo Delivery of AAV for Gene Therapy. Mol. Ther. Methods Clin. Dev. 2, 15041.
53. Szczurekowska, J., Cwieto, A.W., al Maschio, M., Ghezzi, D., Bato, G.M., and Cancedda, R. (2016). Targeted in vivo genetic manipulation of the mouse or rat brain by in utero electroporation with a triple-electrode probe. Nat. Protoc. 11, 399–412.
54. Federici, T., Taub, J.S., Baum, G.R., Gray, S.J., Grieger, J.C., Matthews, K.A., Handy, C.R., Passini, M.A., Samulski, R.J., and Boulis, N.M. (2012). Robust spinal motor neuron transduction following intrathecal delivery of AAV9 in pigs. Gene Ther. 19, 852–859.
