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Supplemental Information

Rapid evolution of blood-brain-barrier-penetrating AAV capsids by RNA-driven biopanning

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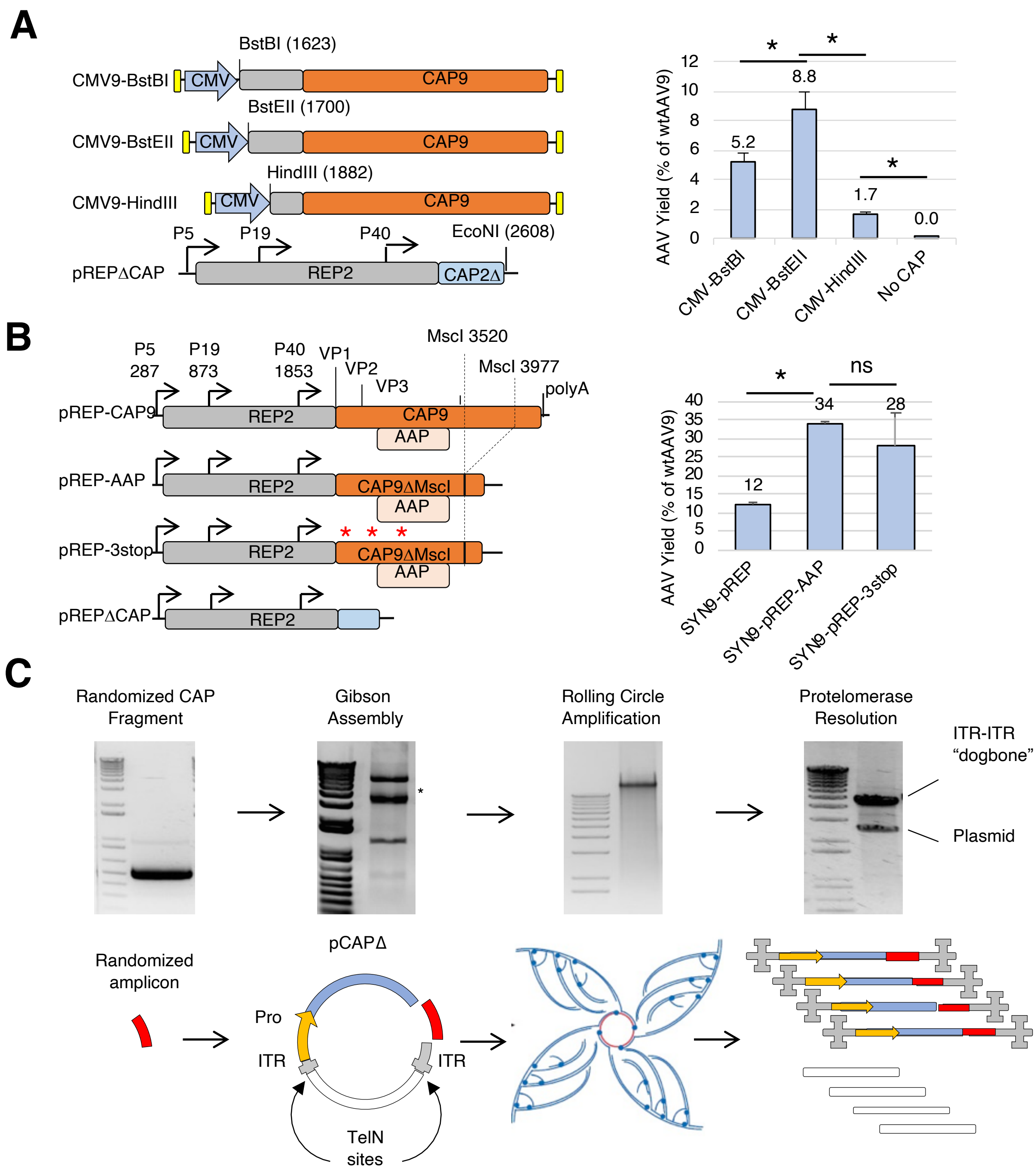


Figure S1 . TRACER library design and optimization.
(A) Identification of the minimal *cis* sequences necessary for efficient AAV production. Variable 5' REP sequences from ITR-REP-CAP9-ITR wild-type vector were replaced by a CMV promoter. The REP protein was provided in *trans* by the pREPΔCAP vector (depicted). Genome titers obtained with each construct are shown on the right bar graph. Values indicate mean ± SD (n=3) percent of wtAAV9 titers. *p < 0.05 (unpaired t test). (B) Optimization of REP vector. Top: map of the parent REP2CAP9 construct showing the position of AAV promoters, CAP ORF start codons and the MscI truncations. The pREP-3stop vector contains nonsense mutations downstream of each capsid ORF start codon (red asterisks). Each REP plasmid was used to produce an ITR-SYN-CAP9-ITR vector (not pictured). The bar graph represents the mean ± SD (n=3) percentage of wtAAV9 genomic titers. *p < 0.05 (unpaired t test); ns, not significant.(C) High-diversity library generation by cloning-free rolling circle amplification. See materials and methods for details.

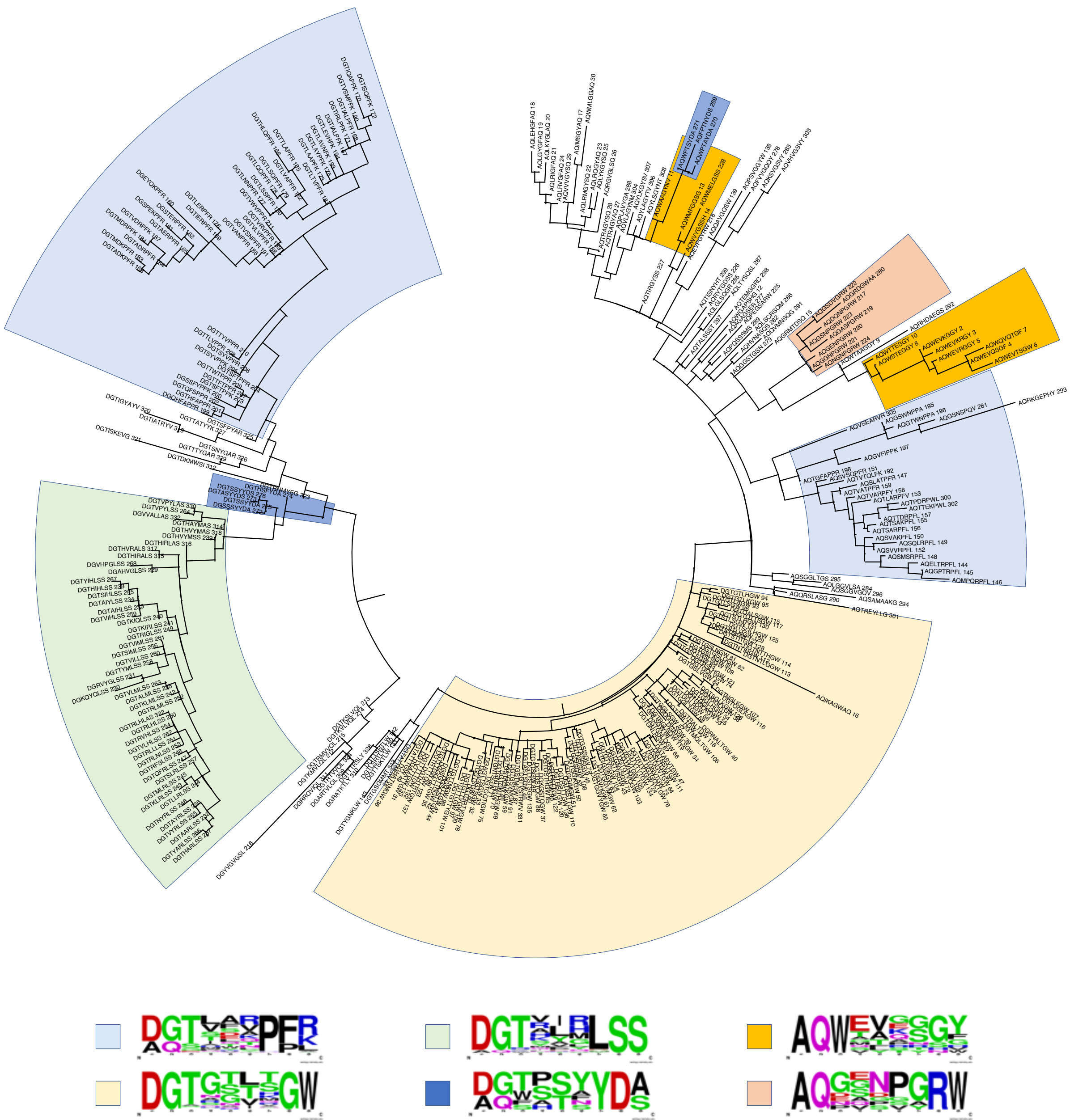


Figure S2. Phylogenetic Analysis of 330 Top BBB-Crossing Variants.
Maximum-likelihood phylogeny relating 330 top variants from mouse brain RNA enrichment analysis. Phylogenic tree of 9-mer variable peptide inserts was constructed using MEGAX. Clusters of peptide sequences sharing high homology are highlighted. Frequency plots of each major cluster are shown at the bottom. The DGTxxxPF[+] consensus motif is shared with PHP.B and PHP.eB capsids and referred to as “PHP-like” in the main text.

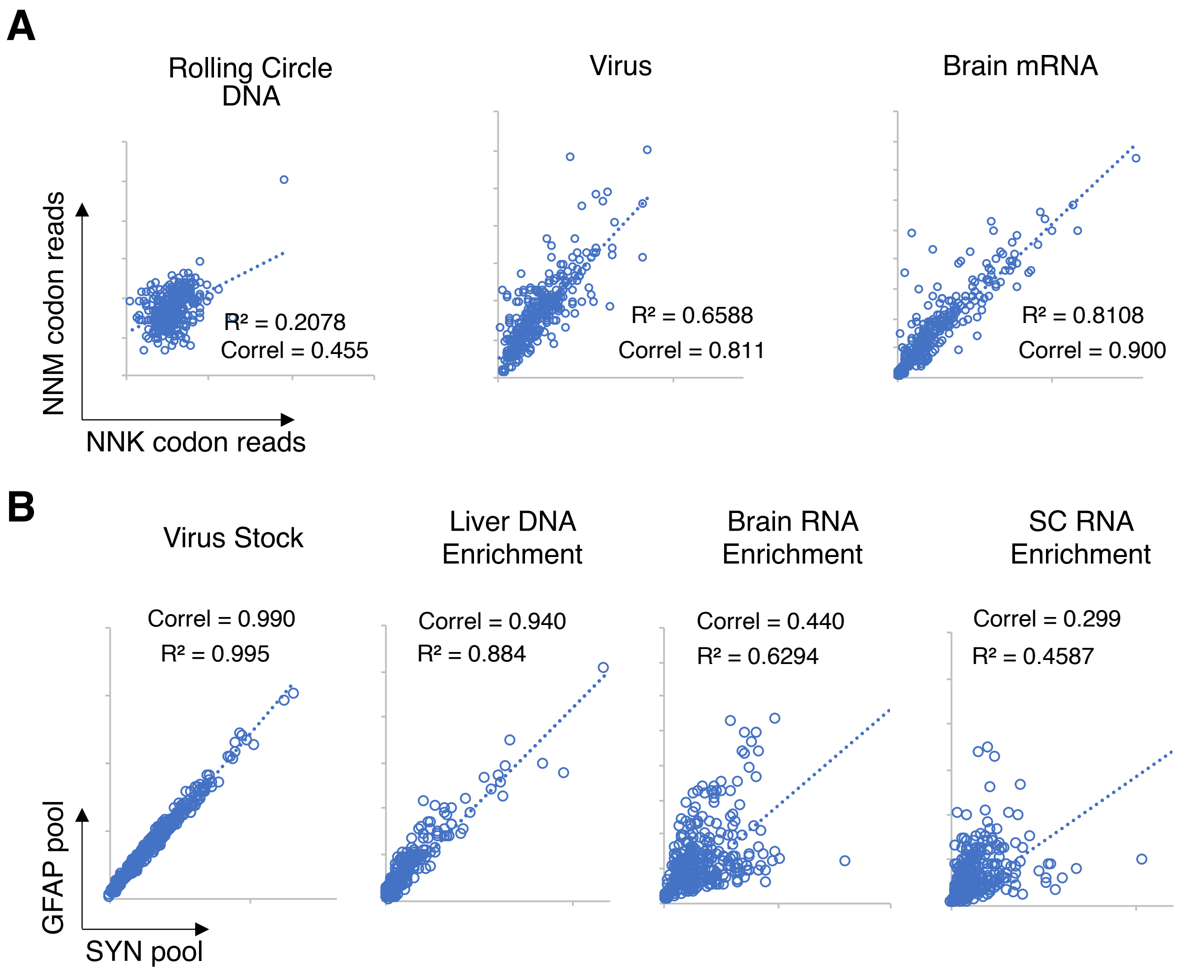
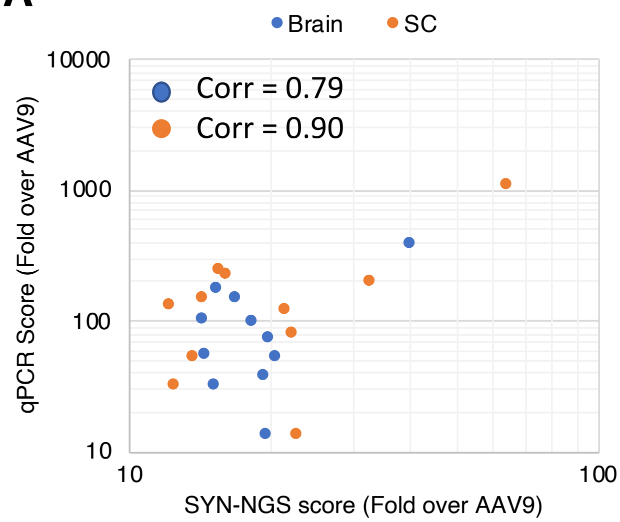


Figure S3 . Correlation analysis of synthetic pooled library.
(A) Codon variant correlation. Scatter plots indicate normalized NGS reads from NNM codon variants (Y axis) and NNK codon variants (X axis) of each capsid mutant in the Rolling circle DNA (left), the virus preparation (middle) and the brain mRNA samples recovered from C57Bl/6 mice (right). Both axes are in linear scale. (B) Correlation analysis of SYN- and GFAP-driven capsid pools in the virus stock, liver DNA, brain RNA and spinal cord RNA. Both axes are in linear scale. Note the high correlation in liver DNA suggesting consistency of both libraries.

A



B

qPCR / NGS	Brain	S.C.	Heart	Liver
SYN Brain	0.79			
SYN SC		0.90		
SYN Heart			0.16	
SYN Liver				0.38
GFAP Brain	-0.07			
GFAP SC		-0.31		
GFAP Heart			-0.22	
GFAP Liver				0.16

Figure S4. Correlation analysis between multiplexed NGS analysis and individual capsid qPCR quantitation. (A) Scatter plot showing the score of each capsid variant as measured by multiplexed brain RNA enrichment (X axis) or individual qPCR RNA quantitation (Y axis) in the brain (blue dots) and spinal cord (orange dots). Values from both assays are normalized to AAV9 for consistency. The correlation coefficients are indicated. (B) Correlation coefficients between qPCR data (rows) and SYN- or GFAP-driven NGS data (columns) of 12 capsids (10 candidates + PHP.eB + AAV9) in the brain, spinal cord, heart and liver. Note the high correlation coefficient obtained with the SYN-driven TRACER data.

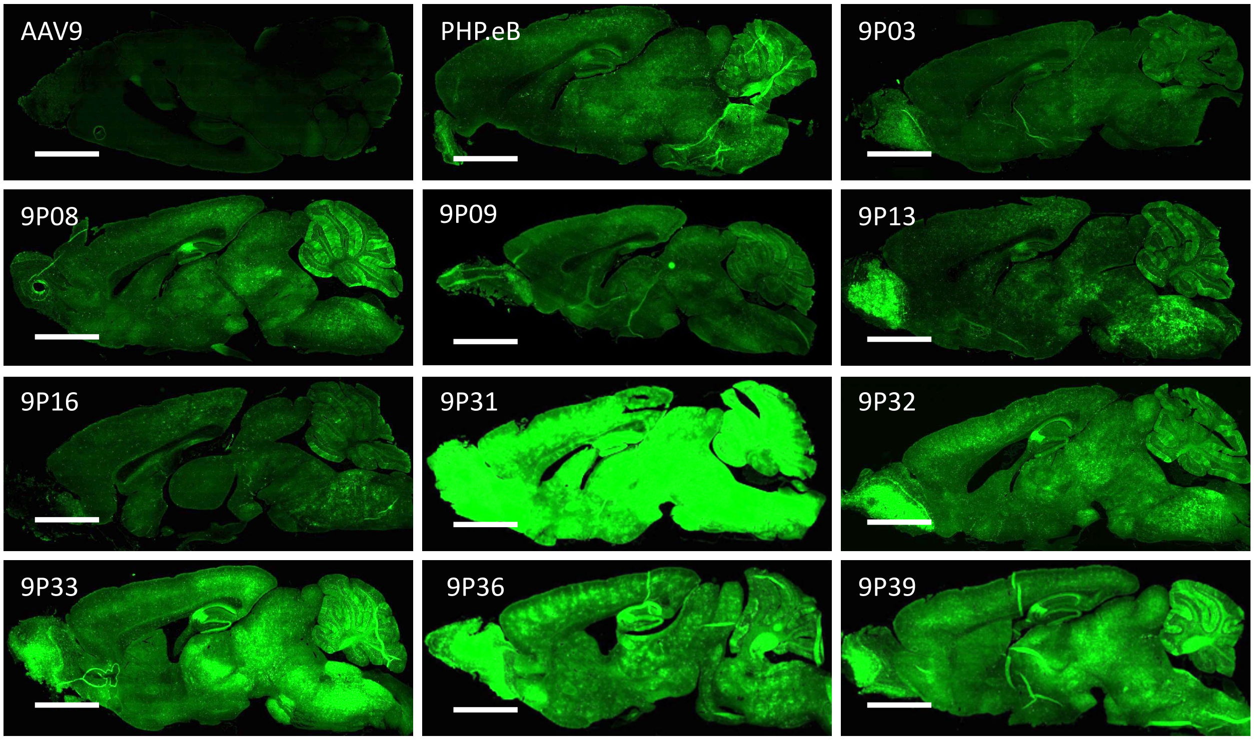


Figure S5. General brain transduction profile of TRACER capsid candidates after intravascular infusion in adult mice.
Native EGFP fluorescence was observed in sagittal cryosections from the brain of adult C57Bl/6 mice 28 days after dosing with 4e11 VG per mouse. Bar: 2 mm.

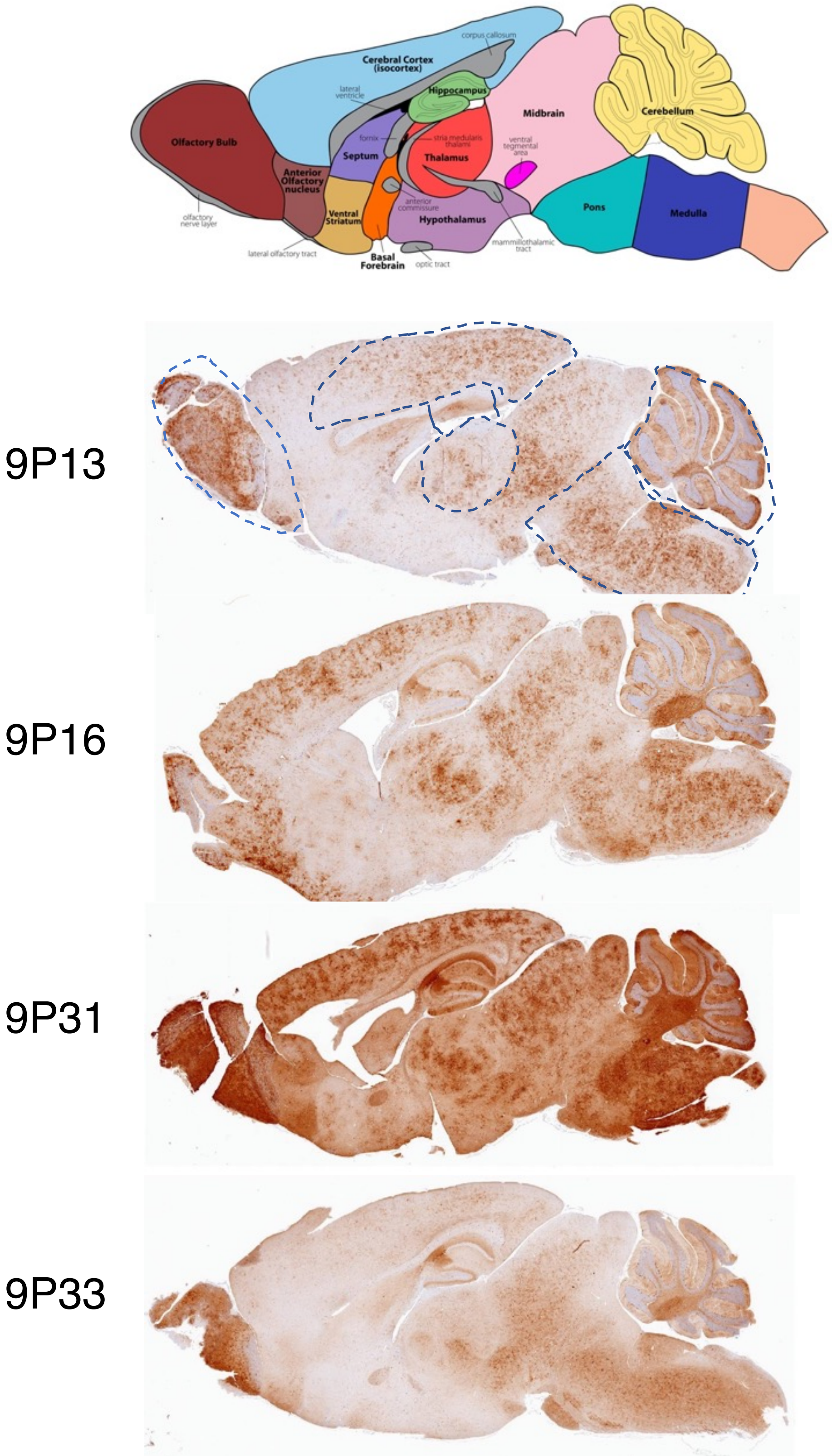


Figure S6. Representative pattern of transduction by TRACER capsids in mouse brain.
EGFP IHC from mouse brains 28 days after intravascular infusion with 9P13, 9P31 and 9P33 capsids (4e11VG per mouse). Major brain regions are depicted in the top diagram (image from http://www.gensat.org).

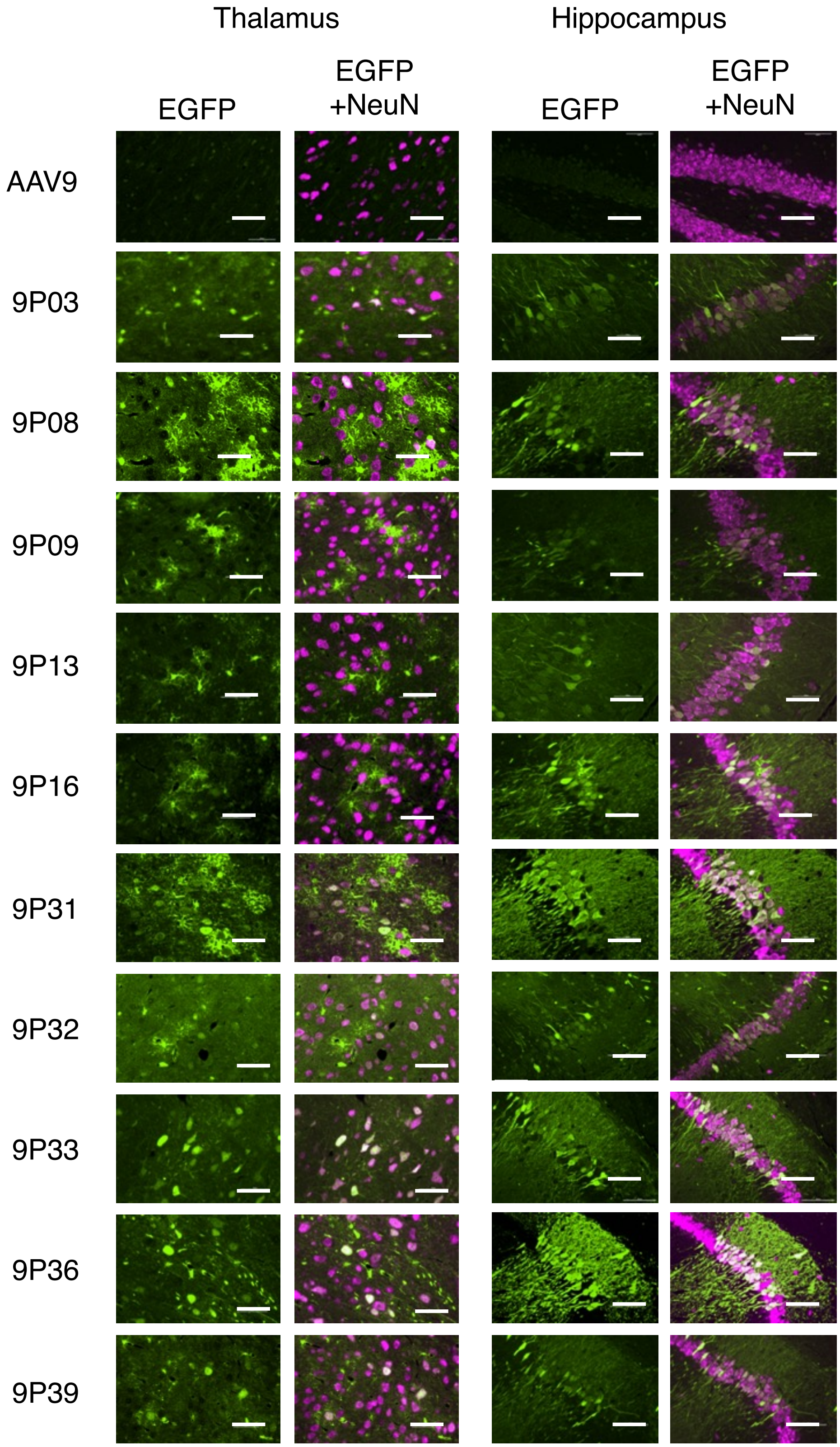


Figure S7. Neuron transduction by TRACER capsids in mouse Thalamus and Hippocampus.
Co-immunostaining of EGFP (green) and NeuN (magenta) in the brain of mice one month after intravenous dosing with 4e11 VG of engineered capsids. Bar: 50 µm.

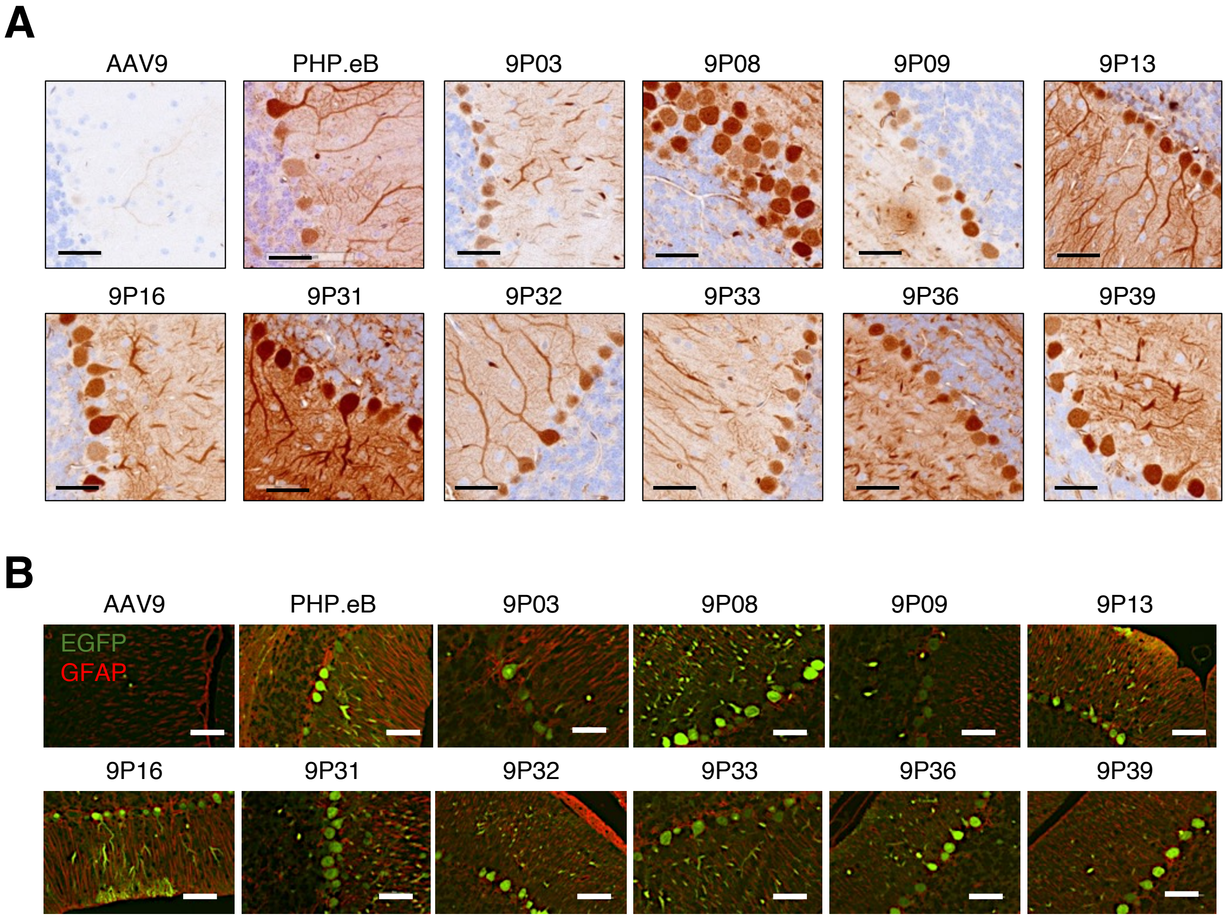


Figure S8. Transduction by TRACER capsids in mouse cerebellum.
(A) Detail of EGFP immunostaining in mouse cerebellum 28 days after intravascular infusion (4e11VG per mouse).
(B) Co-immunostaining of EGFP (green) and GFAP (red) in the cerebellum. Bar, 50 µm.

Supplemental Table 1. Primers and probes used in this study

Primer name	Primer sequence (5′ to 3′)
9L8-F24	CAAGTGGCCACAAACCACCAGAGTgcccaaNNKNNKNNKNNKNNKNNKNNKGCACAGGCGCAGACCGGCTG
9DGL8-F24	CAAGTGGCCACAAACCACCAGAGTgatggcNNKNNKNNKNNKNNKNNKNNKGCACAGGCGCAGACCGGCTG
9DGTL8-F24	CAAGTGGCCACAAACCACCAGAGTgatggcaccNNKNNKNNKNNKNNKGCACAGGCGCAGACCGGCTG
CAP9-L8F	CAAGTGGCCACAAACCACCAGAGT
CAP9-StopR23	CGGTTTATTGATTAACAATCGATTACAGATTACGAGTCAGGTATC
CAP9L8 gBlock[a] ΔBamHI ΔAfeI	GCACAGGCGCAGACCGGCTGGGTTCAAAACCAAGGAATACTTCCGGGTATGGTTTGGCAGGACAGAGATGTGTACCTGCAAGGACC CATTTGGGCCAAAATTCCTCACACGGACGGCAACTTTCACCCTTCTCCGCTGATGGGAGGGTTTGGAATGAAGCACCCGCCTCCTC AGATCCTCATCAAAAACACACCTGTACCTGCcGATCCTCCAACGGCCTTCAACAAGGACAAGCTGAACTCTTTCATCACCCAGTAT TCTACTGGCCAAGTCAGCGTGGAGATCGAGTGGGAGCTGCAGAAGGAAAACAGCAAGCGgTGGAACCCGGAGATCCAGTACACTTC CAACTATTACAAGTCTAATAATGTTGAATTTGCTGTTAATACTGAAGGTGTATATAGTGAACCCCGCCCCATTGGCACCAGATACC TGACTCGTAATCTGTAA
SpliceF6[b]	GTGCCAAGAGTGAC/CTCCTG
CAP-RT	GAAACGAATTAAACGGTTTATTGATTAACAATCGATTA
9*NGS-F4N	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNTGGCCACAAACCACCAGAGT
9*NGS-F3N	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNTGGCCACAAACCACCAGAGT
9*NGS-F2N	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNTGGCCACAAACCACCAGAGT
9*NGS-R[c]	CAAGCAGAAGACGGCATACGAGAT(nnnnnn)GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGTTTTGAACCCAGCCGGT
REP-Fwd2	TTTCCGGTGGGCAAAGG
REP-Rev2	GCTCACTTATATCTGCGTCACT
REP-Probe	ACGTGGTTGAGGTGGAGCATGAAT
GloX4-F	GGGAACGGTGCATTGGAA
GloX4-R	GATGGGCCAGCACACAG
GloX4-Probe[b]	AAGAGTGAC/CTCCTGGGCAACG
EGFP	Life Technologies Mr04329676_mr Taqman set
Mouse TBP	Life Technologies Mm01277042_m1 Taqman set
Human GAPDH	Life Technologies Hs01922876_u1 Taqman set
Mouse TERT	Life Technologies Mm00653609_cn Taqman set

[a]Silent mutations have been introduced to remove BamHI and AfeI sites
[b]Specific for CMV-Globin Exon-Exon junction, does not work on DNA
[c]Bracketed 6-mer represents the site if insertion of illumina TruSeq index for multiplexing

VECTOR SEQUENCES

TRACER-SYN-9-BsrGI (6827bp)

Features:
17-161:   Left ITR
207-763:   Human Synapsin 1 promoter
784-1349:   CMV-Globin hybrid intron
1372-1857: `AAV2 REP C-terminal sequence
1875-3631: AAV9 CAP Fragment
3632-3637: BsrGI restriction site
3762-3906: Right ITR
4008-4063: TelN recognition sequence
4718-5575: Ampicillin Resistance
6577-6632: TelN recognition sequence

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TRACER-GFAP-9-BsrGI (6827bp)

Features:

17-161: Left ITR
207-905: GFAbc1D promoter
926-1491: CMV-Globin hybrid intron
1514-2000: `AAV2 REP C-terminal sequence
2017-3773: AAV9 CAP Fragment
3774-3779: BsrGI restriction site
3904-4048: Right ITR
4150-4205: TelN recognition sequence
4860-5720: Ampicillin Resistance
6719-6774: TelN recognition sequence

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pREP-3stop

Features:

68-1933: AAV2 REP ORF
1950-3703: AAV9 CAPΔ Fragment
1965-1967: Premature VP1 stop codon
2376-2378: Premature VP2 stop codon
2595-2597: Premature VP3 stop codon
3520-3525: MscI restriction site
5207-6067: Ampicillin Resistance

GTCGACGGTATCGGGGGAGCTCGCAGGGTCTCCATTTTGAAGCGGGAGGTTTGAACGCGCAGCCGCCATGCCGGGGT
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scAAV-CAG-EGFP

Features:

1-105:	Left trsΔITR
138-796*f*:	CMV/CBA Promoter
817-1382:	Hybrid CMV-Globin intron
1404-2135:	EGFP
2143-2211:	TK2 polyadenylation sequence
2248-2377:	Right ITR
3140-4000:	Ampicillin Resistance

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