A rationally evolved AAV capsid achieves superior parenchymal penetration and efficient Purkinje cell transduction in non-human primates via intrathecal delivery

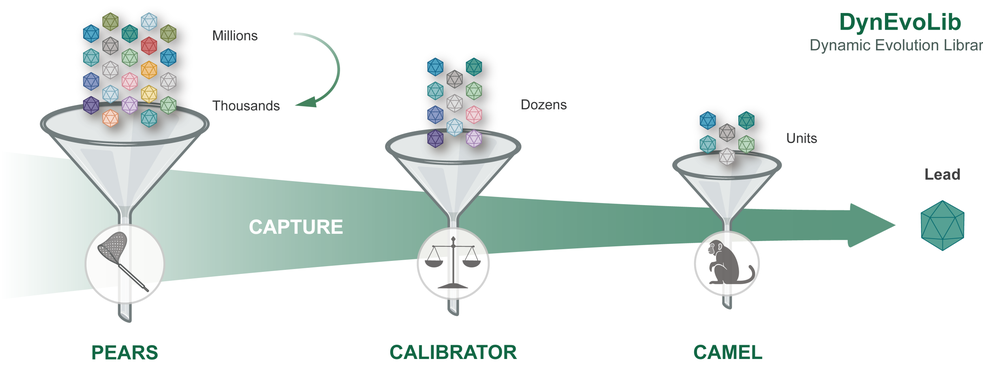
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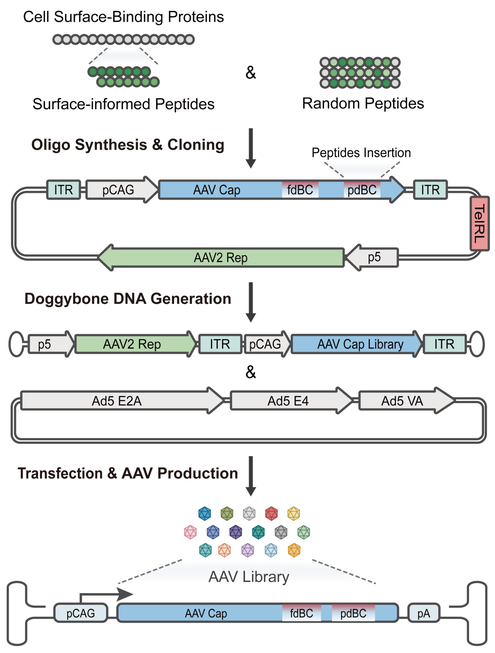
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INTRODUCTION

AAV-based gene therapy holds immense potential for neurological disorders; however, achieving widespread central nervous system (CNS) transgene expression in large-brained species via clinically preferred intrathecal (IT) administration remains a formidable challenge. To address this, we developed DynEvoLib, an integrated capsid engineering platform combining rational design with directed evolution. The platform comprises four key modules (PEARS, CAPTURE, CALIBRATOR, and CAMEL) that work sequentially to enable efficient capsid discovery and validation.



Workflow of DynEvoLib



Schematic of the PEARS module

PEARS (Peptide-driven Evolution of AAV via Randomized and Surface-informed libraries): Integrates randomized and surface-informed peptide libraries (derived from ~15,000 cell surface interaction proteins, yielding a 4-million-variant library) into the AAV9 capsid VR-VIII region via a Dual-DNA Packaging System. Utilizing a dual-barcoding system (fdBC/pdBC), it enables highly efficient in vivo enrichment from millions of variants to thousands of dominant candidates.

CAPTURE (Capsid-Associated Probe-based Transcript Universal Recovery and Enrichment): Utilizes biotin-labeled probe liquid hybridization for highly specific enrichment of viral transcripts from animal tissues.

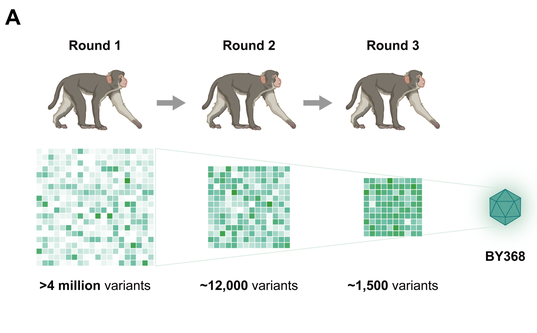
CALIBRATOR (Calibrated AAV Libraries with Internal Barcode-Referenced Analytes for True Output Ranking): Establishes an SCRC (Standard-Curve-based Regression Calibration) model based on AAV9 internal control barcodes to normalize in vivo barcoded pool screening data, enabling true and precise ranking of candidate variants.

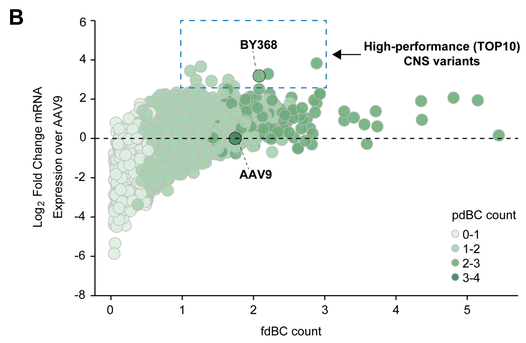
CAMEL (Capsid Assessment via a Multiplexed Epitope-tagging Library): Introduces multiplexed epitope tags into candidate capsids, enabling synchronous evaluation of variants at DNA, RNA, and protein levels within a single animal.

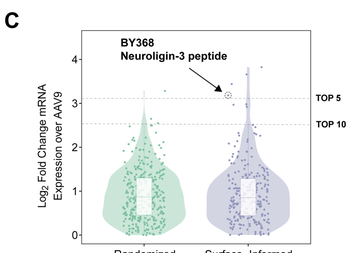
Using this platform, we performed three rounds of non-human primates (NHP) intrathecal screening and identified a novel capsid, BY368, which was further validated through multilevel profiling in rhesus macaques, cross-species validation in cynomolgus macaques, and intracerebroventricular (ICV) delivery in mice.

RESULTS

Directed Evolution of CNS-Targeting Capsids via NHP Intrathecal Screening Identifies BY368





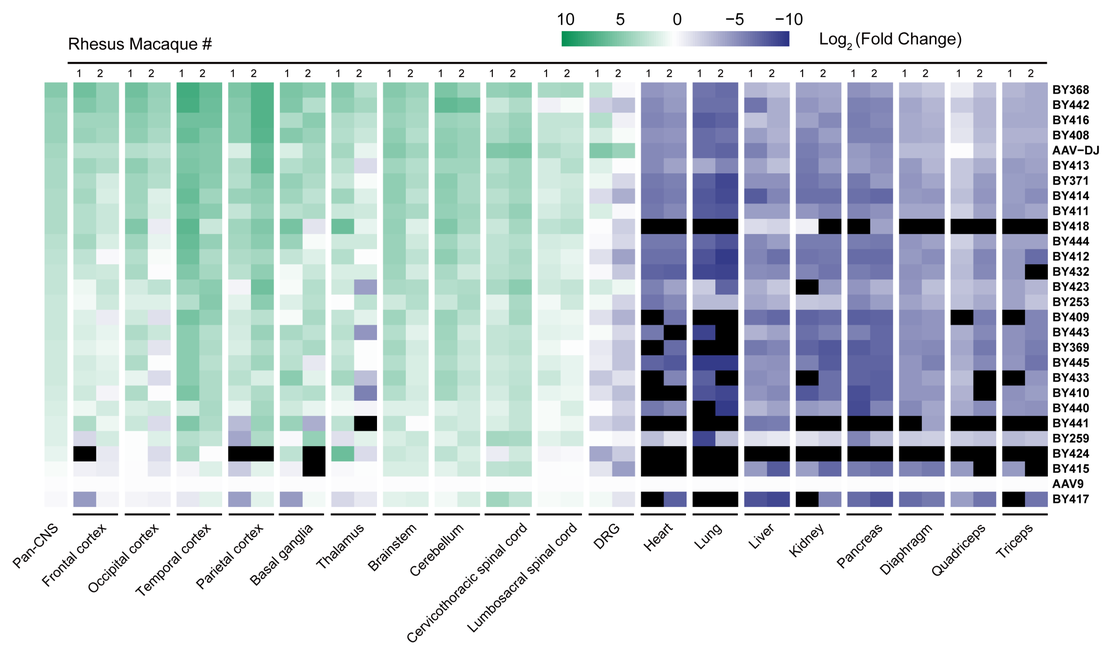


A. Three-Round In Vivo Screening. An initial library (>4 million variants) was intrathecally injected into rhesus macaques. Three rounds of screening enriched it to ~12,000, then ~1,500, identifying BY368.

B. Superior Transduction. The Round 3 library showed significant enrichment across CNS regions. The box highlights TOP10 variants (e.g., BY368) showing high transduction and dual-barcode recovery.

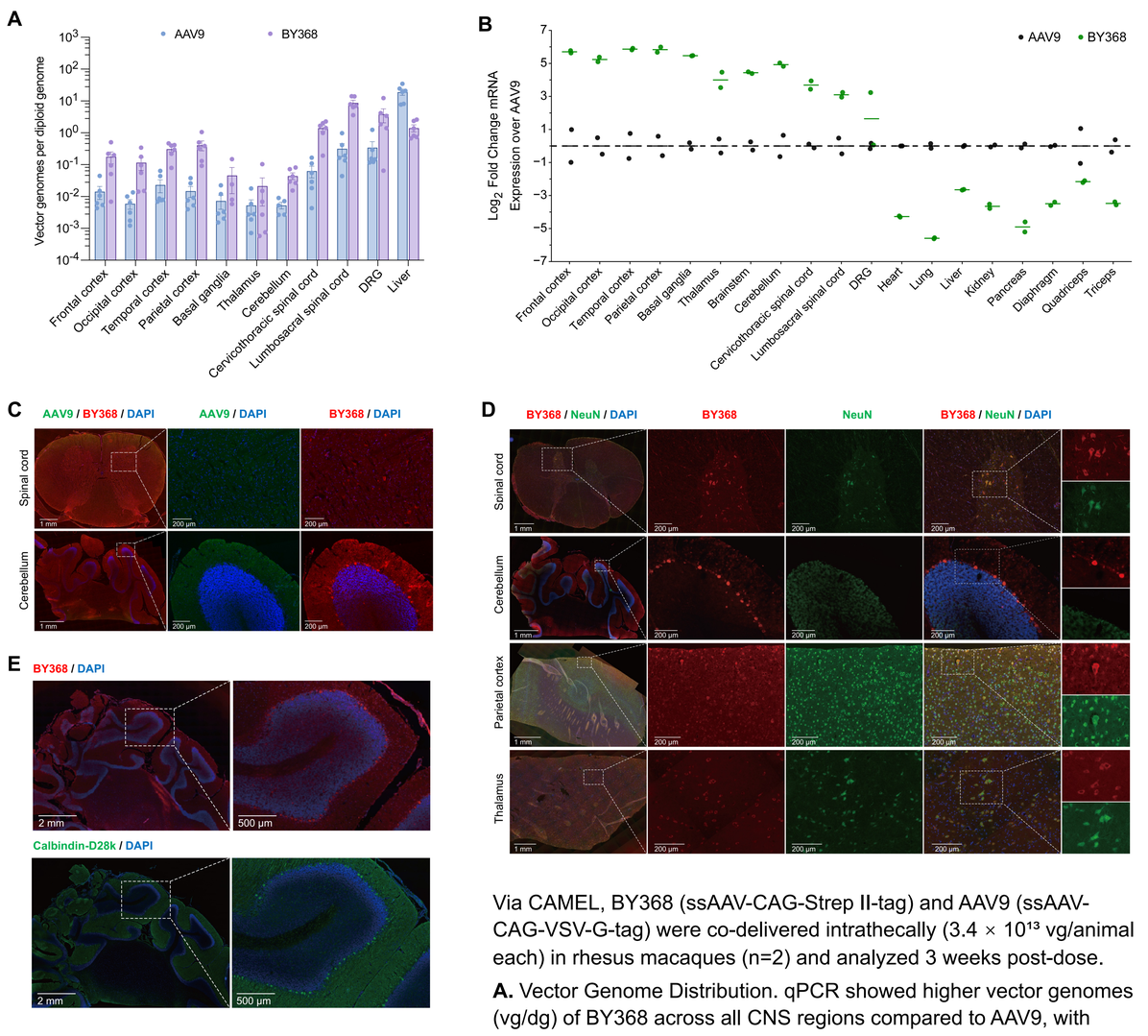
C. Library Source Analysis. While the randomized library contributed 57.4% of overall enhanced variants, the surface-informed library dominated the top performers (70% of Top 10, 80% of Top 5).

Transgene Expression Profiling of Top Candidates via Barcoded Pool Screening



A barcoded pool was intrathecally injected into rhesus macaques (n=2) and analyzed via CALIBRATOR. The heatmap shows relative mRNA expression (Log_2 fold change vs. AAV9) across CNS regions and peripheral tissues, with variants ranked by Pan-CNS (geometric mean of all CNS tissues). Top-ranked BY368 achieved on average 25.71-fold higher brain transduction and 15.51-fold higher spinal cord transduction, with reduced peripheral retention. Additionally, BY442 (TOP2) reduced the dorsal root ganglion (DRG) off-target effects to only 16% of those of AAV9.

Broad CNS Neuronal Transduction and Efficient Purkinje Cell Targeting of BY368 in Rhesus



Via CAMEL, BY368 (ssAAV-CAG-Strep II-tag) and AAV9 (ssAAV-CAG-VSV-G-tag) were co-delivered intrathecally (3.4×10^{13} vg/animal each) in rhesus macaques (n=2) and analyzed 3 weeks post-dose.

A. Vector Genome Distribution. qPCR showed higher vector genomes (vg/dg) of BY368 across all CNS regions compared to AAV9, with lower accumulation in the liver. Data represent n=2 animals, with 3 technical replicates per tissue (6 data points total), plotted as individual dots with mean ± SD.

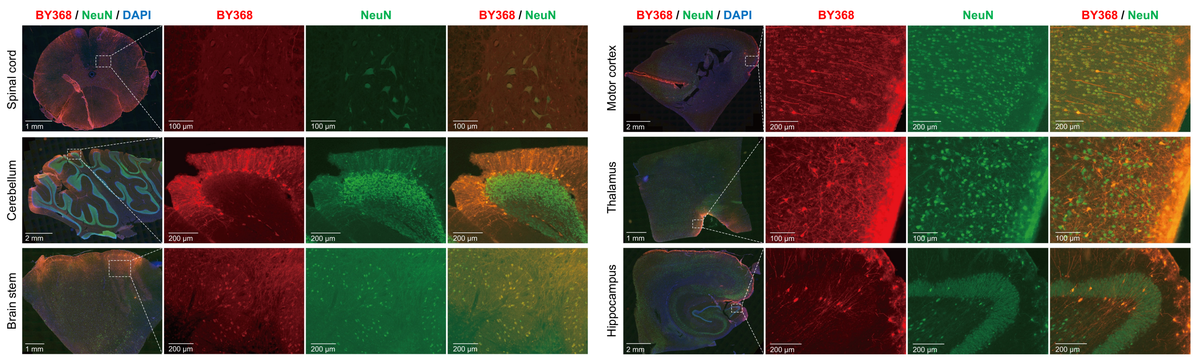
B. Transcriptional Expression. RNA-seq showed that BY368 mRNA was on average 36.32- and 10.51-fold higher in the brain and spinal cord, respectively, compared to AAV9, with low peripheral expression (e.g., the heart, lung, and liver).

C. Protein Expression. Immunofluorescence confirmed widespread and stronger protein expression of BY368 (red) in the spinal cord and cerebellum of rhesus macaques compared to AAV9 (green).

D. Neuronal Co-localization. BY368 (red) showed extensive co-localization with the mature neuron marker NeuN (green) in the spinal cord, cerebellum, cortex, and thalamus.

E. Purkinje Cell Targeting in the cerebellum. Due to the weak NeuN expression in mature Purkinje cells, serial sections were stained with BY368 and Calbindin-D28k. The strong BY368 signal aligned with the Calbindin-D28k-positive Purkinje layer, confirming efficient Purkinje cell transduction.

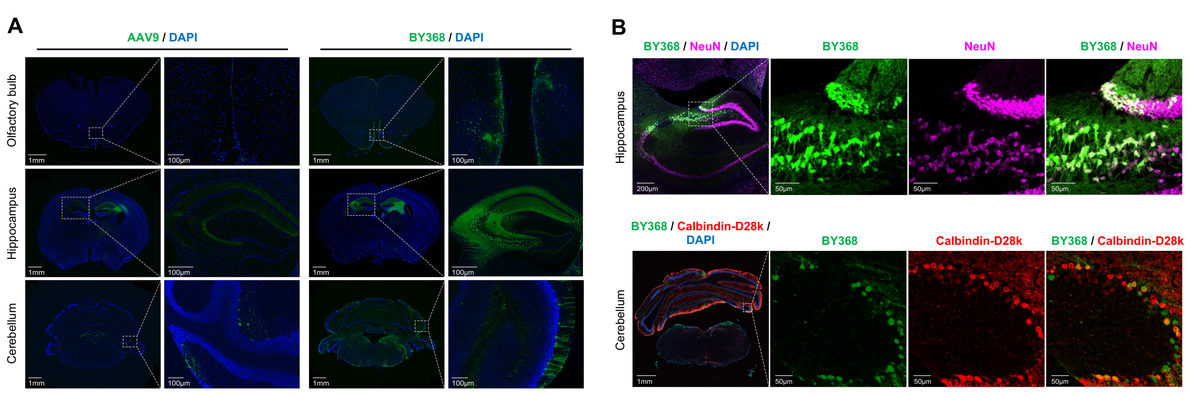
Parenchymal Penetration and Broad Neuronal Tropism of BY368 in Cynomolgus



IT delivery of BY368 (ssAAV-CAG-mCherry) at a dose of 1.9×10^{13} vg/animal in cynomolgus macaques (n=2), analyzed via immunofluorescence (IF) 2-3 weeks post-dose.

BY368 (red) not only transduced cells at the meningeal surface, but also achieved extensive parenchymal penetration, reaching deep structures across multiple CNS regions (spinal cord, cerebellum, brain stem, motor cortex, thalamus, hippocampus). Within these regions, mCherry expression extensively co-localized with the mature neuron marker NeuN (green), indicating efficient neuronal transduction and broad neuronal tropism.

BY368 Mediates Superior CNS Transduction Following ICV Delivery in Mice



ICV delivery of AAV9 or BY368 (ssAAV-CAG-eGFP) at 1×10^{11} vg/mouse in adult C57BL/6 mice (n=4/group), analyzed 3 weeks post-dose.

A. Native GFP fluorescence images showed that, compared to AAV9, BY368 exhibited widespread expression in the olfactory bulb, strong bilateral transduction with deep parenchymal penetration in the hippocampus, and robust distal transduction in the cerebellar cortex.

B. IF staining revealed that BY368 (green) efficiently transduced NeuN+ neurons (magenta) in the hippocampus and Calbindin-D28k+ Purkinje cells (red) in the cerebellum.

CONCLUSION

- We developed DynEvoLib, an integrated AAV capsid engineering platform. Through three rounds of NHP intrathecal screening, we identified BY368, displaying a human Neuroligin-3-derived peptide, as a top CNS-targeting capsid.
- In rhesus macaques (IT delivery), BY368 achieved ~30-fold higher overall CNS mRNA expression than AAV9, with broad neuronal tropism, efficient Purkinje cell targeting, and reduced peripheral transduction (e.g., liver and heart).
- In cynomolgus macaques (IT delivery), BY368 demonstrated parenchymal penetration and broad neuronal tropism across multiple CNS regions. These findings were further confirmed in mice (ICV delivery).
- BY368 represents a highly potent CNS-targeted AAV capsid with a favorable systemic safety profile, making it a promising candidate for CNS-targeted gene therapy.