CSF delivery of Anc80L65 in the nonhuman primate brain results in widespread gene transfer throughout the central nervous system compared to AAV9

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Anc80L65 demonstrates superior expression profile in the CNS compared to AAV9

Current limitations with AAV

- Therapeutic application of AAV has been limited by poor distribution throughout the CNS and inefficient transduction of target cell populations

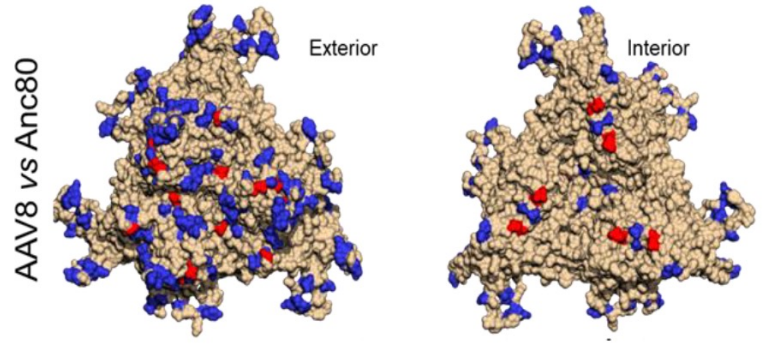
Anc80L65 solution

- We designed a novel AAV capsid, Anc80L65 based on ancestral sequence reconstruction with sequence information from natural AAVs
- A single injection of Anc80L65 in adult cynomolgus monkeys led to more efficient transduction of broad regions of the CNS compared to AAV9 by either lumbar puncture (LP) injection into the lumbar cistern (approximately L3-L4) or intracisternal magna (ICM) injection

Implications

- The ability of Anc80L65 to mediate efficient expression in neurons and astrocytes across many regions of the NHP brain following a single CSF injection has broad implications for treatment of a wide range of neurologic disorders using a relatively noninvasive method of delivery making Anc80L65 a potential candidate for clinical applications

Anc80L65 is 64 amino acids divergent from closest known relative



	AAV5	AAV2	AAV8	rh10	Anc80
AAV5	*ID*	43.0%	42.6%	43.0%	**40.5%**
AAV2	320	*ID*	17.1%	15.9%	**12.2%**
AAV8	317	127	*ID*	6.5%	**9.1%**
rh10	320	118	48	*ID*	**8.6%**
Anc80	**301**	**91**	**68**	**64**	*ID*

Zinn E, et al. Cell Rep. 2015 Aug 11;12(6):1056-68

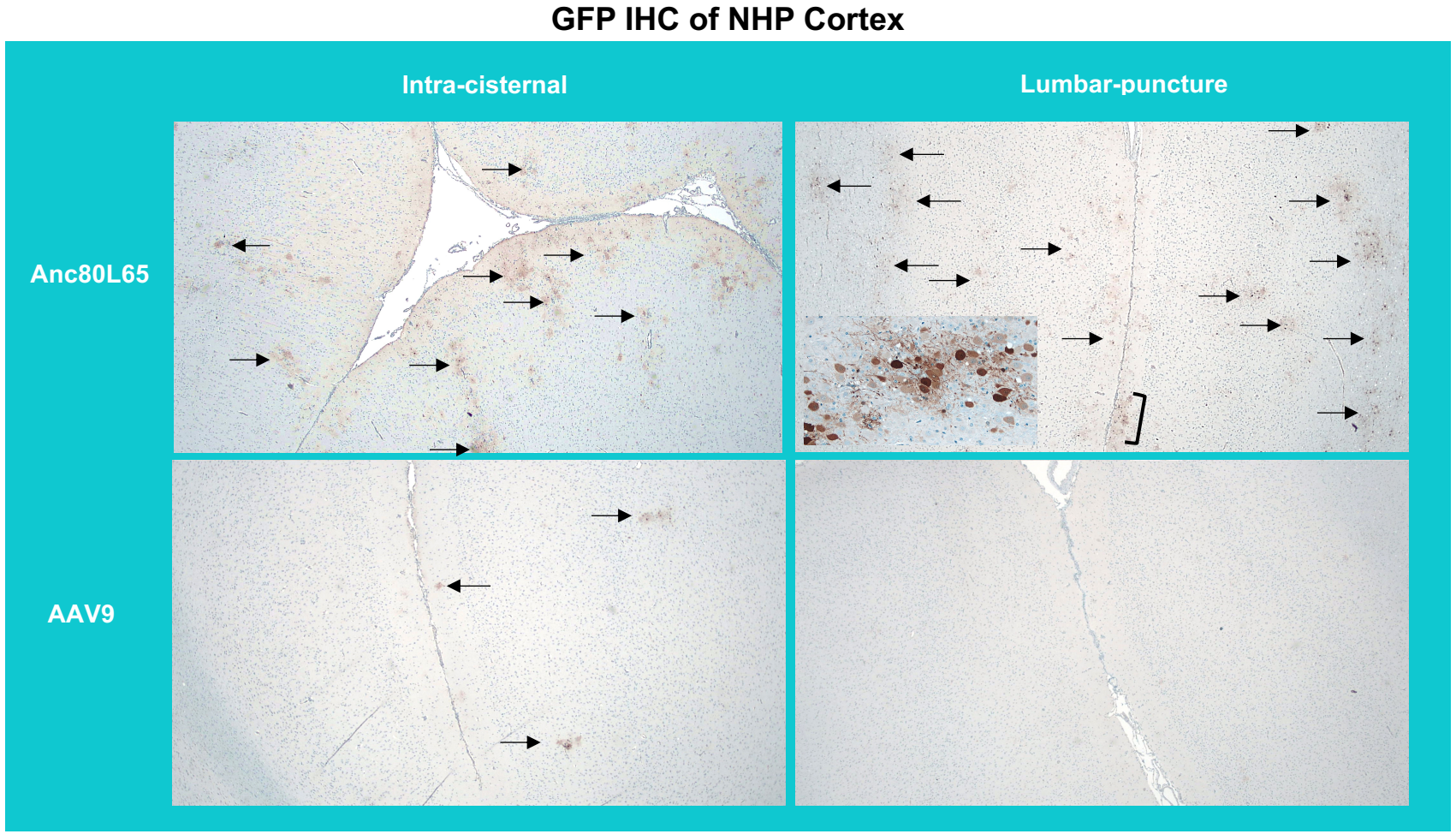
NHP experiment was designed to compare Anc80L65 to AAV9

- **Animals**: Adult male cynomolgus macaques
- **Vectors**: Recombinant AAV vectors Anc80L65 and AAV9-eGFP were produced by triple transfection of suspension grown HEK293 cells and polished using CsCl density gradient centrifugation
- **Delivery**: Animals received an AAV infusion by either lumbar puncture (LP) injection into the lumbar cistern (L3-L4) or intracisterna magna (ICM) injection (4e13 gc/animal; 2ml per infusion, 2e13 vg/ml)

Treatment	Route of Delivery	Dose (vgs)	No of animals	Study duration	Necropsy	Read Outs
Vehicle control	ICM	0.00E+00	1	14 days	Organ collection Brain Spinal cord DRGs Sciatic nerve Kidney Liver Lung Spleen Pancreas Lymph Node Skeletal Muscle	Immunohistochemistry (IHC) GFP NeuN GFAP Iba1 Olig2 ddPCR GFP Expression Vector Genome Copy Number
Anc80L65-CAG-GFP	ICM	4.00E+13	3			
AAV9-CAG-GFP	ICM	4.00E+13	3			
Vehicle control	IT-LP	0.00E+00	1			
Anc80L65-CAG-GFP	IT-LP	4.00E+13	3			
AAV9-CAG-GFP	IT-LP	4.00E+13	3			

Detailed Methods Included in Supplement

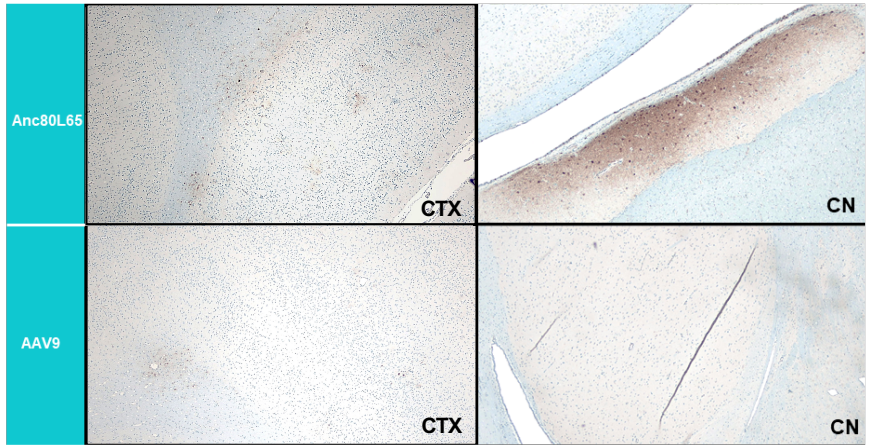
Anc80L65 extensive transduction profile in cortex compared to AAV9 by LP or ICM injection



Low-power (2x objective) view of GFP-immunostained NHP Cortical tissue sections showing Anc80L65 and AAV9 transduction throughout the cortex. GFP-immunostained cell bodies (arrows) and dendritic processes are found throughout the frontal and motor cortex. Inset = 20x objective view (shown by bracket)

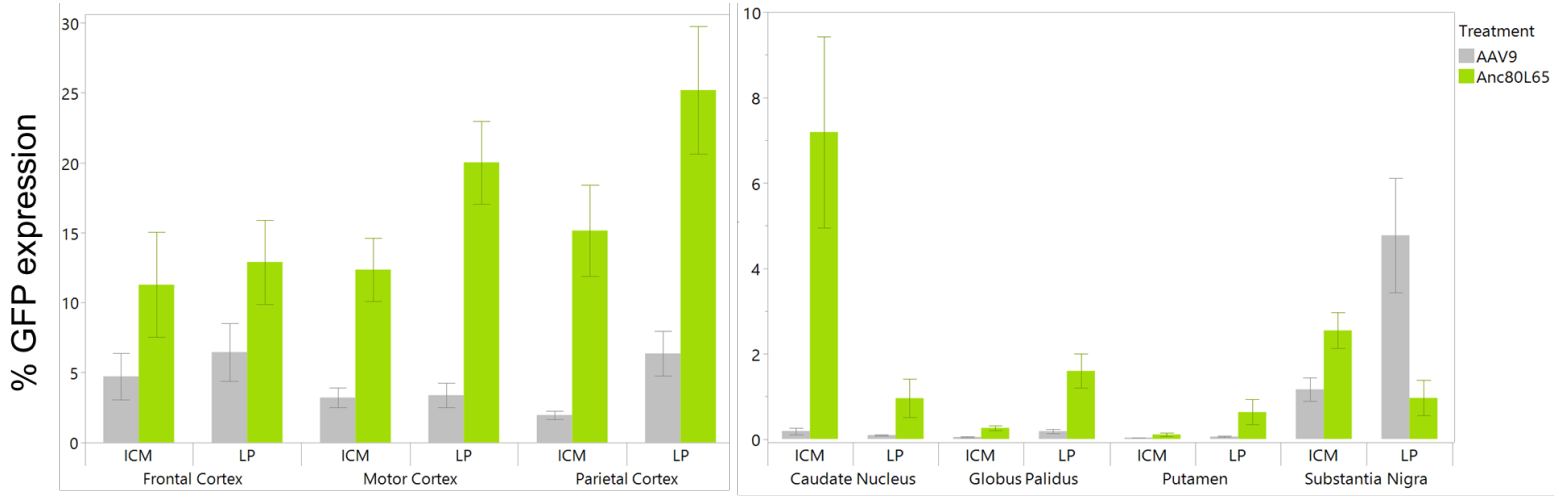
Anc80L65 leads to greater GFP expression throughout the NHP cortex and deep brain nuclei compared to AAV9 irrespective of route of delivery

Expression of eGFP in the NHP brain 2 weeks following ICM delivery



4x objective view of GFP-immunostained NHP tissue showing Anc80L65 and AAV9 transduction in NHP cortex (CTX) and caudate nucleus (CN)

GFP mRNA expression by ddPCR in the NHP brain and spinal cord 2 weeks after ICM or LP delivery

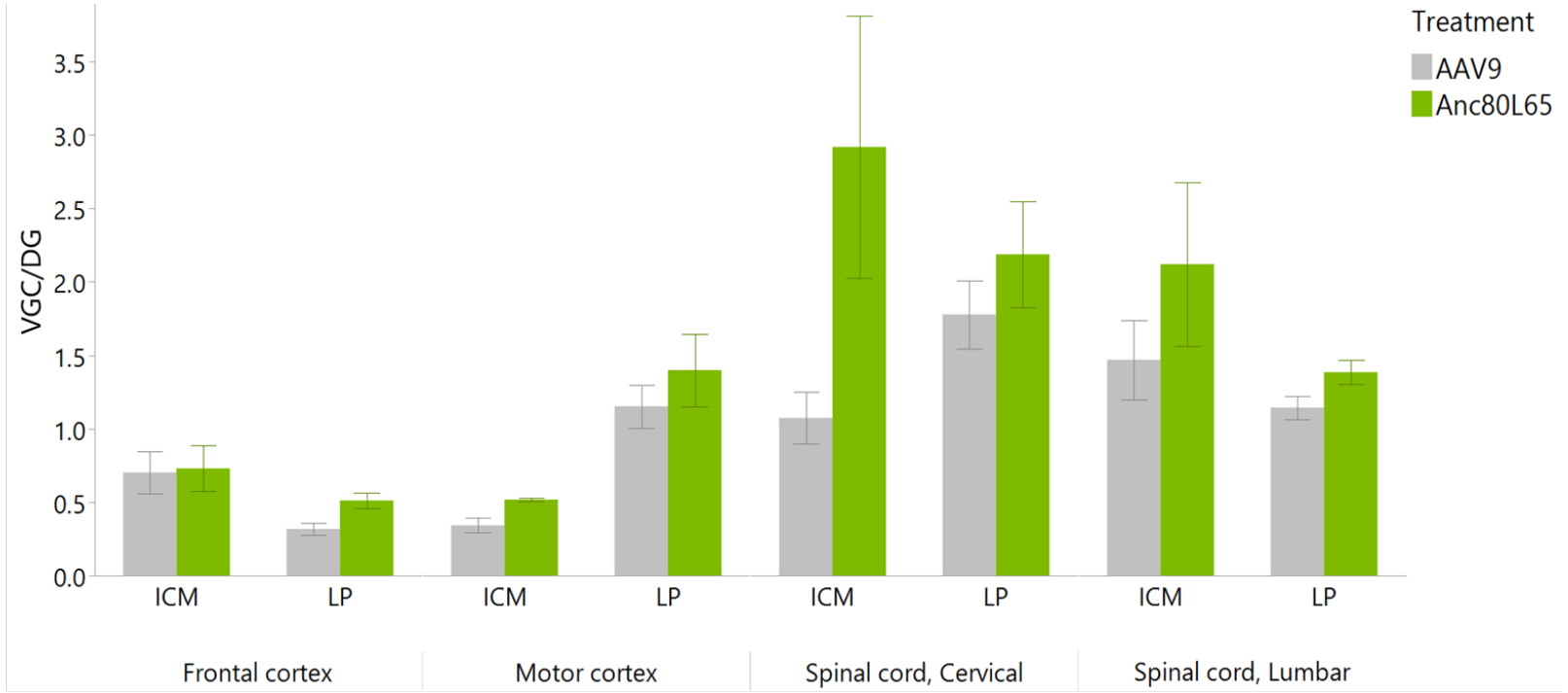


Anc80L65/AAV9 fold-difference	2.4	2.0	3.8	3.0	7.7	4.0	39.9	10.6	5.2	8.8	3.7	12.6	2.2	0.2
p value	0.1379	0.0991	0.001	< 0.0001	0.0037	0.003	0.014	0.0912	0.0046	0.0076	0.1073	0.0888	0.0163	0.0255

qPCR analysis of cortical and deep brain regions and two administration routes (presented as eGFP expression as a percentage of RPP30 expression), showing significantly higher GFP expression in Anc80L65 injected animals compared with AAV9 for both injection routes

Anc80L65 leads to more vector genome copies per cell in NHP brain and spinal cord compared with AAV9 irrespective of injection route

Vector genome copy analysis via qPCR

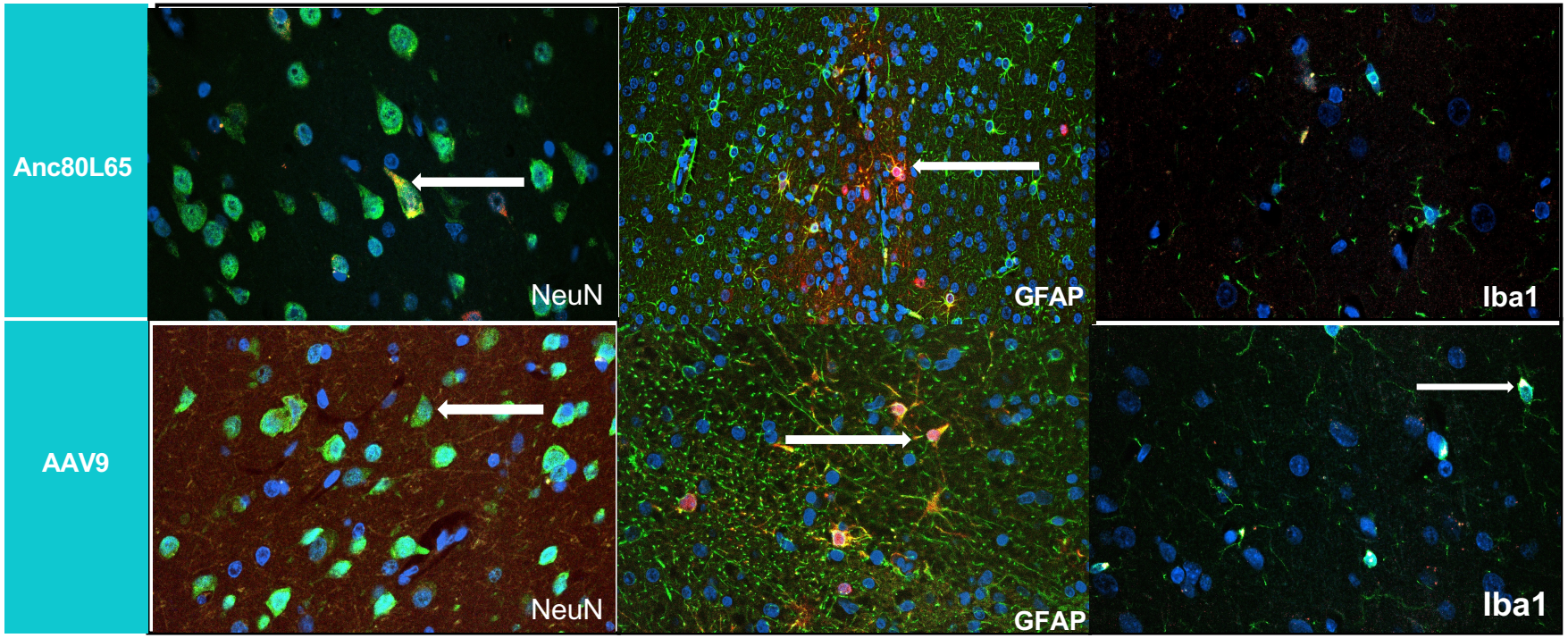


Anc80L65/AAV9 fold-difference	1.0	1.6	1.5	1.2	2.7	1.2	1.4	1.2
p value	0.8981	0.011	0.008	0.406	0.074	0.357	0.316	0.053

VGCs per cell (presented as mean vector genome copies per diploid genome VGC/DG) in NHPs injected with Anc80L65 and AAV9-eGFP by LP or ICM injection

Anc80L65 co-localizes with neuronal and glial cell markers in NHP brain following CSF routes of delivery

Double-labeled IF of Anc80L65 transduced neurons, astrocytes and microglia



Double-labeled IF of Anc80L65 transduced neurons (GFP+/NeuN+), astrocytes (GFP+/GFAP+), and microglial cells (GFP+/iba1+). Examples were imaged from the motor cortex. In all cases, GFP+ cells are shown in red, the cell specific marker is shown in green, and the merged images are shown with double-labeled cells in yellow/orange.

Conclusions and future directions

- A single injection of Anc80L65, a rationally designed synthetic vector, into the CSF of adult cynomolgus monkeys led to more efficient transduction of broad regions of the CNS and strikingly outperformed the capabilities of AAV9 to target the cortex and deep brain nuclei
- A single CSF injection of Anc80L65 distributes more broadly throughout the cortex and into deep brain nuclei compared to AAV9 delivered with either ICM or LP injection
- Anc80L65 distribution by LP injection throughout the cortex was on par with ICM delivery, while AA9 showed little to no transduction in the cortex following the LP route of delivery
- ICM and LP delivery of both Anc80L65 and AAV9 led to robust transduction of the spinal cord and ventral horn motor neurons
- **The ability of Anc80L65 to mediate efficient expression in neurons and astrocytes across large regions of the NHP brain following a single LP injection has broad implications for treatment of a wide range of neurologic disorders using a relatively noninvasive method of delivery making Anc80L65 a potential candidate for clinical applications**
- Quantification of percent transduction across regions and analysis of double-labeled IF to determine co-localization of Anc80L65 with oligodendrocytes is currently being evaluated

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Supplement

Abstract

- **CSF Delivery of Anc80L65 in Nonhuman Primates Results in More Widespread Gene Transfer Throughout the Central Nervous System Compared to AAV9**
  Adeno-associated virus (AAV) gene transfer is a powerful therapeutic modality for treating a number of neurologic disorders. Engineering novel AAV vectors is an exciting emerging area to broaden its reach by addressing the poor distribution throughout the CNS and inefficient transduction of target cell populations using conventional serotypes. The novel AAV vector Anc80L65 was designed based on ancestral sequence reconstruction with sequence information derived from known extant AAVs. Anc80L65 is 101 residues (more than 10%) divergent from AAV9 and was shown to be superior to AAV9 at targeting the brain and spinal cord after intravenous injection in mice and distributed more broadly throughout the CNS. Recent process optimization has significantly improved manufacturing yields ofAnc80L65. These data warranted further testing of Anc80L65 in larger animals. Here, we evaluated distribution of single-stranded AAV9 and Anc80L65 encoding the EGFP reporter 14 days following injection by either lumbar puncture (LP) injection into the lumbar cistern (approximately L3-L4) or intracisterna magna (ICM) injection(4e13 gc/animal; 2e13 vg/ml) in adult cynomolgus macaques. We demonstrate that a single injection of Anc80L65 into the CSF of adult cynomolgus monkeys led to the efficient transduction of broad regions of the CNS. Following ICM injection, Anc80L65 distributes more broadly throughout the cortex and into deep brain nuclei compared to AAV9. Following LP injection, Anc80L65 distribution throughout the cortex was on par with ICM delivery and superior to that seen with AAV9 via ICM delivery. AAV9 showed limited transduction in the cortex following LP delivery. AAV9 and Anc80L65 efficiently transduced spinal cord ventral horn motor neurons with both routes of administration. Quantification of transduction across regions as well as tropism in neurons vs. glial cells are currently being evaluated. This work demonstrates the ability of Anc80L65, a rationally designed novel AAV, to target widespread regions of the CNS following CSF routes of delivery and outperforms the distribution of AAV9 in targeting cortical and deep brain regions. The ability of Anc80L65 to mediate efficient gene transfer and expression in neurons and astrocytes throughout the brain and spinal cord of NHPs supports further investigation in a wide range of neurologic disorders.

Experimental methods

- **Animals**: 14 adult male cynomolgus macaques were included in this study. Survival time was 14 days after AAV delivery for all the animals.
- **Production of AAV vectors**: Recombinant AAV vectors Anc80L65 and AAV9-eGFP were produced by triple transfection of suspension grown human embryonic kidney carcinoma 293 cells (HEK- 293F). Vector was harvested at 3 days post transfection via addition of salts and detergents for 2.5 hours followed by clarification using a depth filtration train. Concentration and buffer exchange occurred through a TFF/DF operation using flat-sheet membranes and filtrate was processed over an affinity column (GE-AVB for Anc80 and CaptureSelect AAV9 for AAV9 Vectors). The vectors were eluted off using a low pH buffer and immediately neutralized. Post neutralization vectors were polished using CsCl density gradient centrifugation and vectors were then formulated and aliquoted for use in future studies.
- **Vector Infusion**: All animals received an infusion of AAV vector by either lumbar puncture (LP) injection into the lumbar cistern (L3-L4 as confirmed by positive cerebral spinal fluid (CSF) flow) or intracisternal magna (ICM) injection (4e13 gc/animal; 2e13 vg/ml) in adult cynomolgus macaques.
- **Histological analysis of transgene expression**: To assess transgene expression, brain sections were processed for immunohistochemical analysis (IHC). Brains were removed and sectioned coronally into 6-mm blocks. The blocks were targeting the transgene (eGFP post-fixed in buffered paraformaldehyde (4%) for 24 h, washed briefly in PBS and adjusted in a 30% sucrose/PBS solution for cryopreservation. The formalin-fixed brain blocks were cut into 40-µm coronal sections in a cryostat. Free-floating sections spanning the entire brain were collected in series and were kept in antifreeze solution for further IHC analysis.
- **Immunohistochemistry**: GFP staining by 3,3'-diaminobenzidine (DAB): Sections (3 per each 6-mm block: separation of 2 mm) were washed 3 times in PBST followed by treatment with 1% H2O2. Sections were stained with the primary anti-GFP antibody diluted 1:1000 in Da Vinci Green Diluent as previously described (San Sebastian et al., 2013).
- **Double-immunofluorescence:** Fluorescence immunostaining of different cellular markers (NeuN, GFAP, Iba1) with GFP as previously described (San Sebastian et al., 2013).
- **ddPCR**: Tissues were homogenized in a Qiagen Tissuelyser II (20rps for 2 min) in lysis buffer from the Qiagen Dneasy Blood and Tissue Kit or the Qiagen RNeasy Lipid Tissue Mini Kit following the standard Qiagen protocol. Samples were eluted in 50uL of buffer. Prior to analysis, DNA and RNA concentration and quality were determined using a NanoDrop One, using the nucleic acid (DNA or RNA) program. DNA samples were analyzed for biodistribution of vector genomes using a duplexed ddPCR method targeting the transgene (eGFP) and a reference gene (RPP30). RNA samples were analyzed for expression of the eGFP transgene using a duplexed, one-step RT-ddPCR method) and a reference gene (RPP30).