


METHODOLOGY

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AAV9-Retro mediates efficient transduction with axon terminal absorption and blood–brain barrier transportation

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Abstract

Recombinant adeno-associated viruses (rAAVs), particularly those that permit efficient gene transfer to neurons from axonal terminals or across the blood–brain barrier, are useful vehicles for structural and functional studies of the neural circuit and for the treatment of many gene-deficient brain diseases that need to compensate for the correct genes in every cell in the whole brain. However, AAVs with these two advantages have not been reported. Here, we describe a new capsid engineering method, which exploits the combination of different capsids and aims to yield a capsid that can provide more alternative routes of administration that are more suitable for the wide-scale transduction of the central nervous system (CNS). A new AAV variant, AAV9-Retro, was developed by inserting the 10-mer peptide fragment from AAV2-Retro into the capsid of AAV9, and the biodistribution properties were evaluated in mice. By intracranial and intravenous injection in the mice, we found that AAV9-Retro can retrogradely infect projection neurons with an efficiency comparable to that of AAV2-Retro and retains the characteristic of AAV9, which can be transported across the nervous system. Our strategy provides a new tool for the manipulation of neural circuits and future preclinical and clinical treatment of some neurological and neurodegenerative disorders.

Keywords: Adeno-associated viruses, Retrogradely, AAV9-retro, Across the blood–brain barrier, Neural circuits

Introduction

Viral vectors, particularly those that permit efficient gene transfer to the central nervous system (CNS) from axonal terminals or across the blood–brain barrier, are useful to analyse the structure and function of specific neuronal circuits around the injection site [1–11] and have become one of the most promising therapy tools to deliver therapeutic genes to a distant target area [5,

12]. Compared with traditional retrograde tracers, viral vectors can express genes in specific neuron groups [9, 13] and have been widely used to monitor and manipulate neuronal activities by expressing optogenetic [14, 15], chemogenetic [16, 17] and calcium-sensitive functional probes [18–20]. Some natural and engineered neurotropic viruses exhibit retrograde infection capabilities, such as the pseudorabies virus (PRV) [21], herpes simplex virus (HSV) [22], rabies virus (RABV) [13, 23], lentivirus (LV) [24–27], canine adenovirus (CAV) [6, 28], and adeno-associated virus (AAV) [5, 29–31]. Among them, PRV is highly toxic [4, 32, 33]; HSV and RV can express genes rapidly and have high retrograde labelling efficiency, but they are also toxic to cells, limiting long-term gene manipulation [4, 13, 34, 35]. Rabies virus with double deletion of G/L has reduced the cytotoxicity and gene expression [35]. Lentiviruses

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packaged by modified RVG have high retrograde transport efficiency [25] but may induce host immune responses [36, 37] or lead to tumorigenesis by random insertion into the host genome [38]. CAV-2 has relatively low immunogenicity, large cloning capacity [39, 40], and enhanced retrograde labelling efficiency after compensation of the CAV-2 receptor in input neurons [6] but only moderate levels of gene expression [41] and difficulties in preparation [42].

Recombinant adeno-associated viruses (rAAVs) have become a powerful tool for neural circuit manipulation and gene therapy because they are low-toxic with high-level transgene expression and minimal host immune responses [43–45]. The treatment of many gene-deficient brain diseases must compensate for the correct genes to every cell in the whole brain [44]. Some natural and engineered adeno-associated viruses can transduce most CNS neurons by axon terminal absorption or blood–brain barrier transportation, such as AAV-TT [44], AAV2-Retro [5], AAV9-SLR [12], MNM004 [46], AAV-PHP.eB [47], and AAV-F [48], among others. However, AAVs with these two important features have not been reported. Compared with AAV2, AAV9 may have greater prospects for modification due to the advantages of lower neutralising antibodies in humans [49], higher transduction efficiency in vivo and the ability to cross the blood–brain barrier [50–52]. AAV9 can deliver therapeutic cargo throughout the CNS to partially correct phenotypes in mice models of CNS disease [53], including mucopolysaccharidosis (MPS) IIIB [54], amyotrophic lateral sclerosis (ALS) [55, 56], and Huntington's disease [57], among others, and has been used to human clinical trials for the treatment of spinal muscular atrophy (SMA, ClinicalTrials.gov: NCT02122952). AAV-TT exceeds the distribution abilities of AAV9 and AAVrh10 in the CNS of mice, and can correct the neurological phenotype in a mouse model of mucopolysaccharidosis IIIC [44]. Enhancing the distribution abilities (retrograde labeling) of AAV9 can provide more alternative routes of administration (intracranial or intravenous administration) that are more suitable for the wide-scale transduction of the central nervous system (CNS) and for gene therapy. Therefore, AAV9 variants that provide efficient transduction by axonal spread and across the blood–brain barrier are needed. Inserting the selected sequences from the AAV9 libraries into the capsid of AAV2 has been reported to not increase the transduction efficiency compared with AAV9 variants, and vice versa in most cases [58]. Therefore, it remains unknown whether the peptide segments from AAV2-Retro (AAV2 libraries) can be integrated into the capsid of AAV9 to achieve high-efficiency retrograde labelling and maintain the ability to cross the blood–brain barrier.

Herein, we inserted the 10-mer peptide fragment from AAV2-Retro (AAV2 libraries) into the capsid of AAV9 and compared it with AAV2-Retro in neurotropism. By intracranial and intravenous administration in mice, we found that this new variant, AAV9-Retro, can retrogradely infect projection neurons with efficiency comparable to AAV2-Retro and retain the ability to cross the blood–brain barrier. Our strategy provides a new retrograde tool for the manipulation of neural circuits and future treatment of some neurological and neurodegenerative diseases.

Materials and methods

Animals

Adult male (8–10 weeks old) C57BL/6J (Hunan SJA Laboratory Animal Company) mice were used for all experiments. The mice were housed in the appropriate environment with a 12/12-h light/dark cycle; water and food were supplied ad libitum. All the surgical and experimental procedures were performed in accordance with the guidelines formulated by the Animal Care and Use Committee of Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences.

AAV9 capsid modification

The 10-mer peptide sequence (LADQDYTKTA) from AAV2-Retro (Addgene plasmid # 81070) was inserted between Q588 and A589 of the AAV9 capsid in the pAAV-RC9 vector (BrainVTA Technology Co., Ltd.). The corresponding 30-bp DNA sequence was introduced into the AAV9 capsid by overlap PCR and cloned into the pAAV-RC9 vector via the restriction enzymes BsiWI and NheI (New England Biolabs). The modified AAV9 capsid plasmids (AAV9-Retro) were verified by DNA sequencing.

Recombinant AAV vector production

The various AAV serotype vectors, including AAV2-Retro-CaMKIIa-EGFP, AAV9-Retro-CaMKIIa-EGFP and AAV2-Retro-CaMKIIa-mCherry, were produced by a baculovirus-AAV expression vector system [59] and purified by iodixanol gradient ultracentrifugation [60, 61]. The purified rAAVs were titered by qPCR using the iQ SYBR Green Supermix kit (Bio-Rad) and diluted to 1.0×10^{13} viral particles/mL.

Administration of AAV particles

The stereotactic injection coordinates were selected according to Paxinos and Franklin's *The Mouse Brain in Stereotaxic Coordinates*, 4th edition [62]. The stereotactic coordinates for the VTA were as follows: AP: -3.20 mm; ML: ± 0.45 mm; DV: -4.30 mm from the bregma. The stereotactic coordinates for

CPu were as follows: AP: +0.38 mm; ML: \pm 2.00 mm; DV: -3.50 mm from the bregma. Eight- to ten-week-old C57BL/6 J mice (20–25 g) were used for aav virus injection, and the standard injection process was performed as previously reported [11]. For single VTA site injection, the mice were divided into two groups (N=3 in each group) and 300 nl of rAAV2-Retro-CaMKIIa-EGFP and rAAV9-Retro-CaMKIIa-EGFP viruses were infused into the VTA of each group. For mixed viral injection into the VTA, rAAV2-Retro-CaMKIIa-mCherry and rAAV9-Retro-CaMKIIa-EGFP viruses were mixed at the particle ratio of 1:1 and injected into the VTA at 300 nl. For single CPu site injection, the mice were divided into two groups (N=3 in each group) and 300 nl of rAAV2-Retro-CaMKIIa-EGFP and rAAV9-Retro-CaMKIIa-EGFP viruses were infused into the CPu region of each group. After 3 weeks of virus expression in vivo, the mice were sacrificed using the conventional cardiac perfusion method. EGFP-expressing rAAV9, rAAV9-Retro and rAAV2-Retro (5×10^{11} vg/mouse) was intravenously injected into adult mice respectively, and the mice were sacrificed after 4 weeks of expression.

Slice preparation and imaging

The brains were soaked with 4% paraformaldehyde solution overnight, dehydration was accomplished at 37 °C with 30% sucrose solution, and the brain was sectioned at a thickness of 40 μ m using a frozen section instrument, and the brain slices were retrieved at 200- μ m intervals. The brain slices were washed 3 times with PBS, 5–10 min each time. After DAPI staining (diluted by PBS at 1:3000) for 10–15 min, the brain slices were washed with PBS 2 times and applied neatly on the microscope slides, followed by sealing with 70% glycerol. Imaging was performed using the Leica TCS SP8 confocal microscope (Leica, Germany) or Olympus VS120 Slide Scanner microscope (Olympus, Japan).

Statistical analysis

For cell counting, the images were divided into different brain regions using Adobe Photoshop CS5 according to the Allen Mouse Brain Atlas (<https://www.brain-map.org/>). The EGFP-positive neurons were quantified using ImageJ software, and the cells from the olfactory bulb were not counted. Data analysis was performed using unpaired t-test and GraphPad Prism (version 5.01; San Diego, CA). The data are shown as means \pm SEM. The statistical significance of differences was set at $P < 0.05$. Graphs of virus injection were drawn using Sigma Plot (version 10.0; Systat Software Inc, San Jose, CA).

Results

Efficient retrograde connectivity tracing by the rAAV9-Retro variant

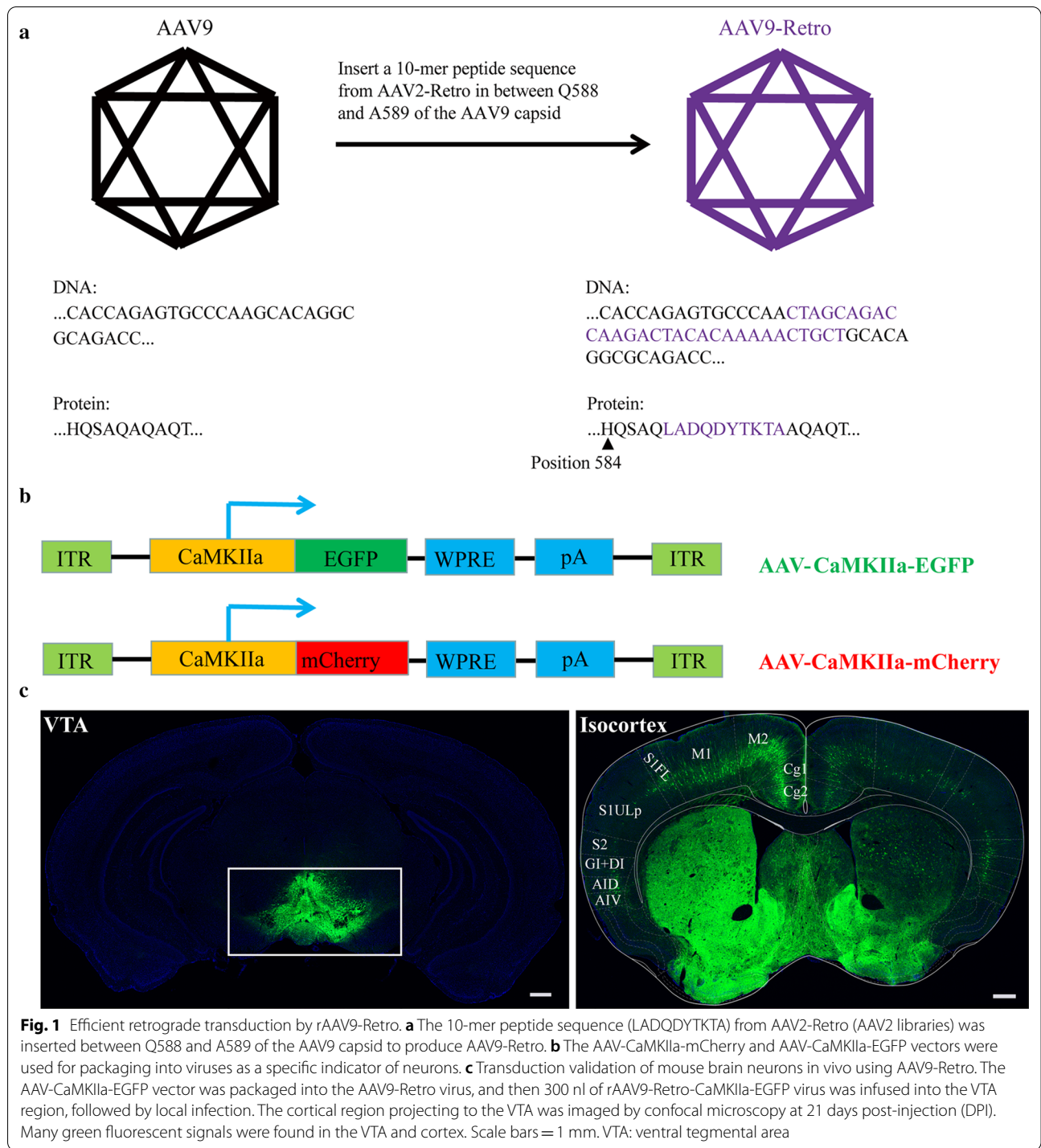
Previous reports have shown that inserting the selected sequences from the AAV9 libraries into the capsid of AAV2 could not increase the transduction efficiency compared with the AAV9 variants [58], and the 7-mer PHP.B peptide (TLAVPFK) from the AAV9 libraries can improve the transduction efficiency of AAV1 in vivo and in vitro when inserted between S588 and T589 of the AAV1 capsid [63]. Therefore, whether the peptide segments from AAV2-Retro (AAV2 libraries) can be integrated into the capsid of AAV9 to achieve high-efficiency retrograde labelling remains to be verified. Thus, the 10-mer peptide sequence (LADQDYTKTA) from AAV2-Retro (AAV2 libraries) was inserted between Q588 and A589 of the AAV9 capsid to produce AAV9-Retro (Fig. 1a). rAAV-CaMKIIa-EGFP and rAAV-CaMKIIa-mCherry were packaged using AAV9-Retro (Fig. 1b). To evaluate whether AAV9-Retro can achieve high-efficiency retrograde labelling input neurons, 300 nl of the rAAV9-Retro-CaMKIIa-EGFP virus was infused into the VTA, and then local infection and the cortical region projecting to the VTA were imaged by confocal microscopy at 21 days post-injection (DPI). Many green fluorescent signals were found in the VTA and cortex (Fig. 1c), indicating that AAV9-Retro has high-efficiency retrograde access to input neurons.

rAAV9-Retro shows a similar retrograde gene transduction efficiency to rAAV2-Retro

rAAV2-Retro has higher retrograde transduction efficiency than AAV9 and plays an important role in analysing nerve circuits [5]. To compare the retrograde infection efficiency of the rAAV9-Retro virus (based on AAV9 modification) with rAAV2-Retro, the two viruses were injected into the VTA sites (Fig. 2A, B). We found that the two viruses could infect the same upstream brain regions (Fig. 2C–J), including the prefrontal cortex (PFC), somatomotor areas (MO), medial septal complex (MSC) and midbrain reticular nucleus (MRN), and no statistical difference was found in the total number of neurons projected into the VTA (Fig. 2K, $10,770 \pm 1394$ for AAV2-Retro; $10,278 \pm 1957$ for AAV9-Retro; $P = 0.8477$). These results indicate that rAAV9-Retro and rAAV2-Retro have similar retrograde infection efficiency.

rAAV9-Retro and rAAV2-Retro can retrogradely label the same projection neurons through mixed injection

To verify whether rAAV9-Retro and rAAV2-Retro can retrogradely infect the same brain regions that project to the injection site and avoid the difference caused by



injection site deviation or individual variation of animals, rAAV2-Retro-CaMKIIa-mCherry and rAAV9-Retro-CaMKIIa-EGFP viruses were mixed at the particle ratio of 1:1 and injected into the VTA at 300 nl (Fig. 3A), and local infection and projection regions were imaged using an Olympus VS120 Slide Scanner microscope at 21 days

post-injection (DPI). Both green and red fluorescent signals appeared in the VTA (Fig. 3B–D) and same brain areas that project into the VTA (Fig. 3E–P), such as the anterior cingulate area (ACA), somatomotor areas (MO), prefrontal cortex (PFC), agranular insular area (AI) and medial septal complex (MSC), with most of them

(See figure on next page.)

Fig. 2 Comparison of the transduction efficiency between rAAV9-Retro and rAAV2-Retro injected into the VTA. **A, B** rAAV2-Retro-CaMKIIa-EGFP and rAAV9-Retro-CaMKIIa-EGFP viruses can efficiently infect neurons when injected into the VTA sites. **C–J** The two viruses can infect the same upstream brain regions, including the PFC, MO, MSC and MRN. **k** Comparison of the total number of neurons projecting into the VTA using rAAV9-Retro and rAAV2-Retro. The data are shown as means \pm SEM. Statistical significance of differences was set at $P < 0.05$. No statistical difference was found in the total number of neurons projecting into the VTA ($10,770 \pm 1394$ for AAV2-Retro; $10,278 \pm 1957$ for AAV9-Retro, $P = 0.8477$). Scale bars = $200 \mu\text{m}$ for Fig A-B; Scale bars = $100 \mu\text{m}$ for figure **C–J**. VTA: ventral tegmental area; PFC: prefrontal cortex; MO: somatomotor areas; MSC: medial septal complex; MRN: midbrain reticular nucleus

overlapping. These results showed that rAAV9-Retro and rAAV2-Retro could retrogradely label the same projection neurons with mixed injection to the same brain area.

rAAV9-Retro and rAAV2-Retro exhibit similar retrograde infection tropism and efficiency at different brain regions

Viruses may have different infective characteristics and efficiency when injected into different brain areas. Therefore, we injected the two viruses into the brain area of the caudate putamen (CPu; Fig. 4A, E), which was reported to mainly receive input from the cerebral cortex (CTX), basolateral amygdalar nucleus (BLA) and thalamus (TH) via rAAV2-Retro [5]. We found that, like rAAV2-Retro (Fig. 4B–D), rAAV9-Retro effectively infected these brain regions (Fig. 4F–H), and the efficiency was equivalent between them through quantitative analysis (Fig. 4I, from CTX: 6495 ± 479.8 for AAV2-Retro, 7119 ± 507.6 for AAV9-Retro, $P = 0.4219$; from BLA: 737.3 ± 19.19 for AAV2-Retro, 871 ± 203.1 for AAV9-Retro, $P = 0.5481$; from TH: 1281 ± 250.0 for AAV2-Retro, 945.0 ± 256.6 for AAV9-Retro, $P = 0.4010$). Additionally, no statistical difference was found in the total number of neuronal projections to CPu (Fig. 4J; 8888 ± 672.8 for AAV2-Retro, 9366 ± 633.6 for AAV9-Retro; $P = 0.6318$). These results indicate that rAAV9-Retro and rAAV2-Retro have similar retrograde infection tropism and efficiency at different brain regions.

rAAV9-Retro mediates efficient transduction across the central nervous systems after intravenous administration

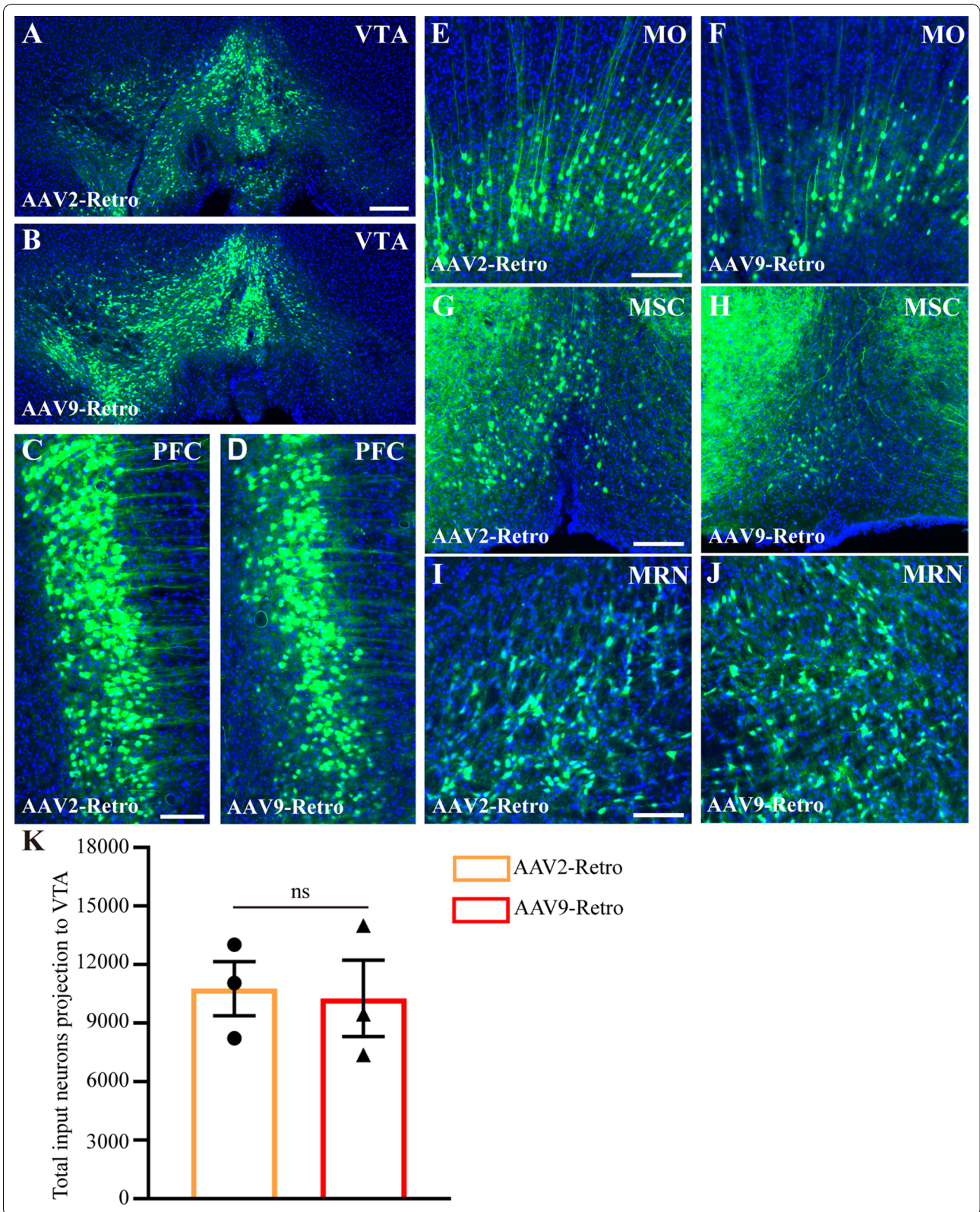
Compared with AAV2, AAV9 has the advantage of transduction across the blood–brain barrier. To verify whether rAAV9-Retro can mediate efficient transduction across the central nervous systems, EGFP-expressing rAAV9, rAAV9-Retro and rAAV2-Retro (5×10^{11} vg/mouse) was intravenously injected into adult mice respectively, and the fluorescence signals in the brains of mice were imaged after 4 weeks of expression. We found that rAAV9-Retro allowed efficient transduction across the blood–brain barrier after intravenous administration (Fig. 5a), and EGFP signals were observed in several important brain regions (including the cortex, striatum, pallidum, hippocampus, thalamus, hypothalamus and

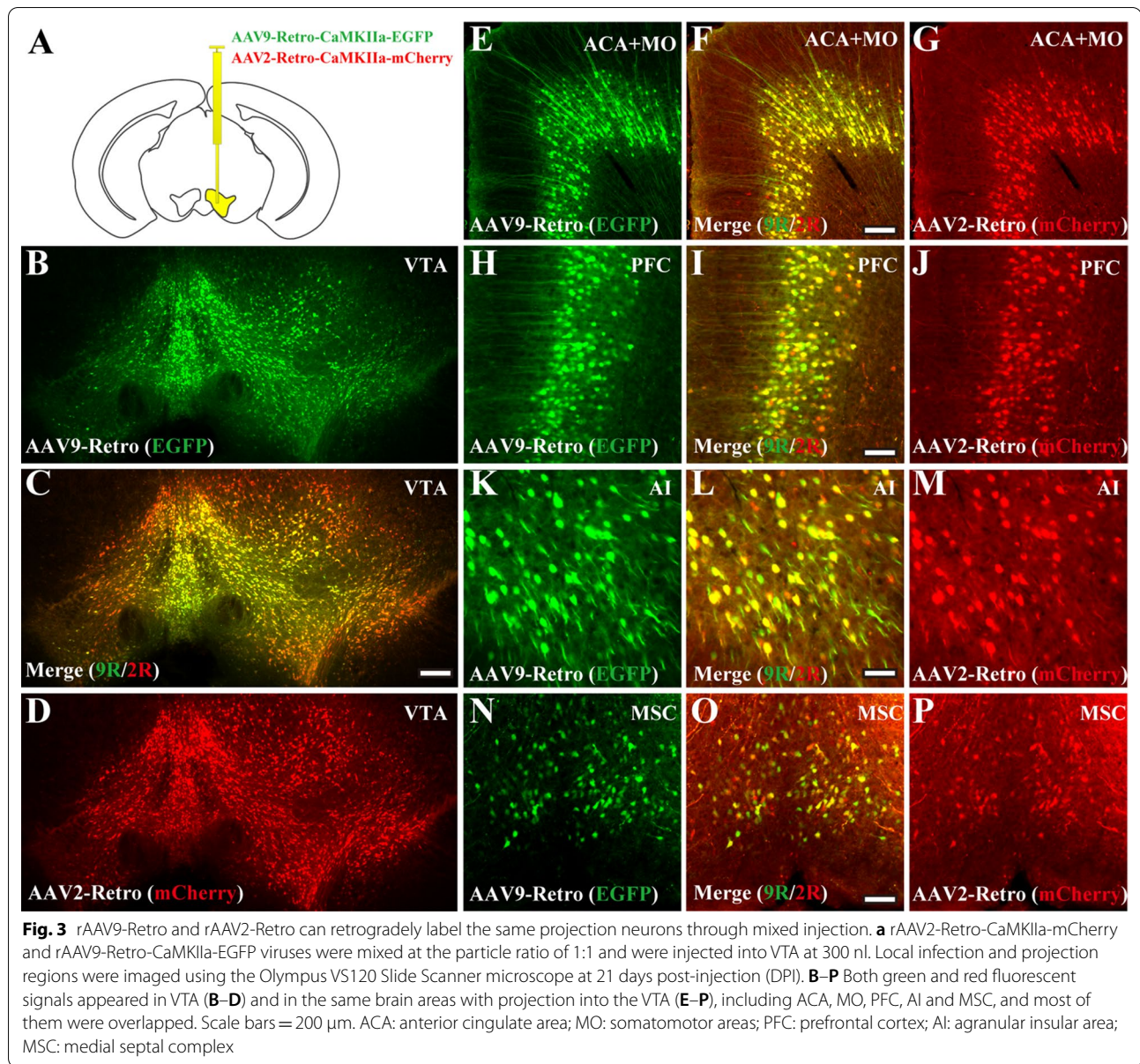
periaqueductal grey), which were not shown in rAAV2-Retro (Fig. 5b). Importantly, no statistical difference between rAAV9 and rAAV9-Retro was found in the total number of EGFP-positive neurons in the brain (Fig. 6, 2323 ± 308.6 for AAV9, 2513 ± 672.2 for AAV9-Retro; $P = 0.8096$). These results indicate that AAV9-Retro can mediate efficient transduction across the CNS with an efficiency comparable to that of AAV9.

Discussion

Adeno-associated viruses play an important role in understanding the structure and function of the brain [64]. They are also the primary vectors in the field of gene therapy and can be used in the treatment of various genetic defects, such as Parkinson's disease [65], haemophilia [66, 67], and lysosomal disease [68]. Herein, we have endowed the AAV9 capsid with efficient retrograde transduction capacity in the mouse brain by inserting the 10-mer peptide sequence (LADQDYTKTA) from AAV2-Retro (AAV2 libraries) between Q588 and A589. The retrograde infection tropism and efficiency of rAAV9-Retro are similar to those of rAAV2-Retro in neural circuits. Importantly, rAAV9-Retro retains the ability to deliver genes across the blood–brain barrier.

Various neurotropic viruses, such as HSV1 [22], CAV2 [6], RABV [13], HiRet and NeuRet lentivirus [25], which have been widely used in the study of brain circuits, have the characteristics of retrograde access to specific or multiple types of neurons [69, 70]. However, high toxicity makes them unsuitable for long-term functional studies in model animals, further limiting their application in disease treatment [4]. Although nontoxic rabies virus has been developed, its expression ability is weaker and needs to expand the expression of foreign genes by carrying CRE and FLP recombinase combined with transgenic animals or AAV viruses [35]. Several AAV variants have been developed for more efficient transduction of the nervous system. For example, rAAV2-Retro allows for the efficient mapping, monitoring, and manipulation of projection neurons [5]. Since the retrograde infection tropism and efficiency of rAAV9-Retro are similar to those of rAAV2-Retro in neural circuits, rAAV9-Retro can also be used for the structural mapping and functional manipulation of input neurons. In addition, due to less toxicity

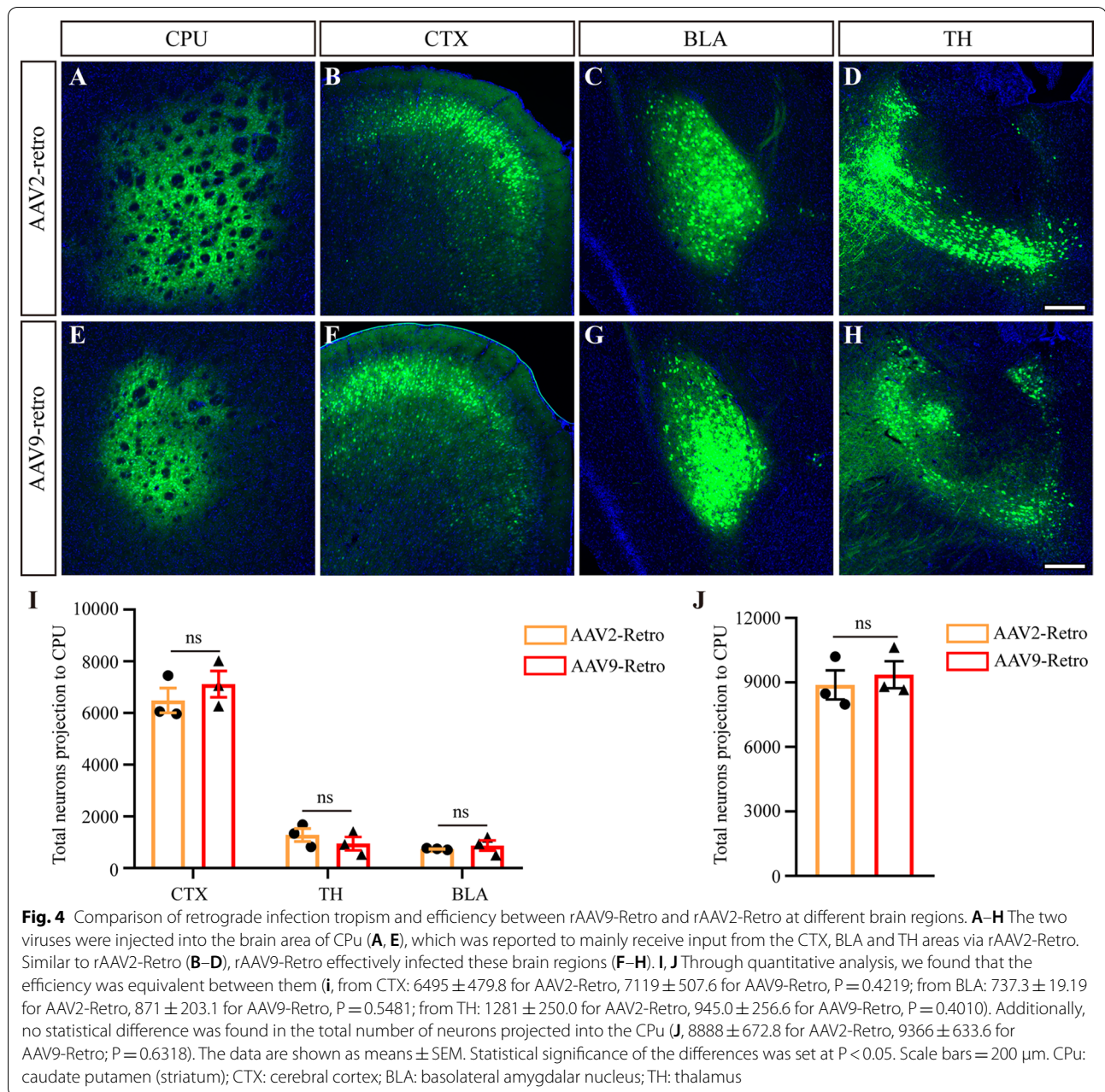




and high-efficiency retrograde labelling, rAAV9-Retro can replace CAV2 in TRIO system (for tracing the relationship between input and output) and cell-type-specific TRIO system (cTRIO) [71], which have been developed to determine the input–output organization of neural circuits [71, 72]. AAV9 has lower neutralising antibodies in vivo, and the AAV9 variant can evade the neutralisation reaction more effectively than AAV2 or AAV2 variant in vitro [49, 58]. In addition, increasing the efficiency of AAV9 transduction and decreasing the administration dosage can reduce the immune response caused by a dose increase [73]. Moreover, compared with AAV2, AAV9 is less affected by the change in the extracellular

matrix in the brain [12]. Therefore, AAV9-Retro may be a better vector for functional network manipulation and gene therapy.

Non-human primates are deal experimental animals for human disease modeling [74, 75], some recombinant adeno-associated viruses (rAAVs) can undergo retrograde transport in non-human primates, but the efficiency of retrograde transport is low, limiting the analysis of functional circuits and implications for disease modeling [76]. AAV2-Retro can mediate extensive retrograde transport in the rhesus macaque brain [77], but whether AAV9-Retro can achieve high-efficiency retrograde labelling in non-human primates brain is

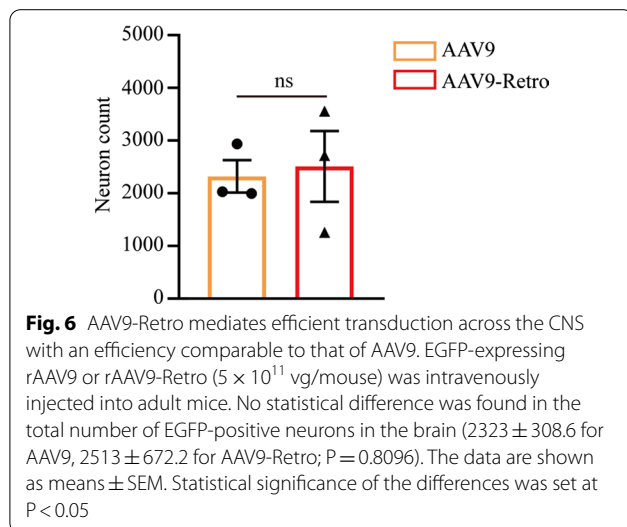
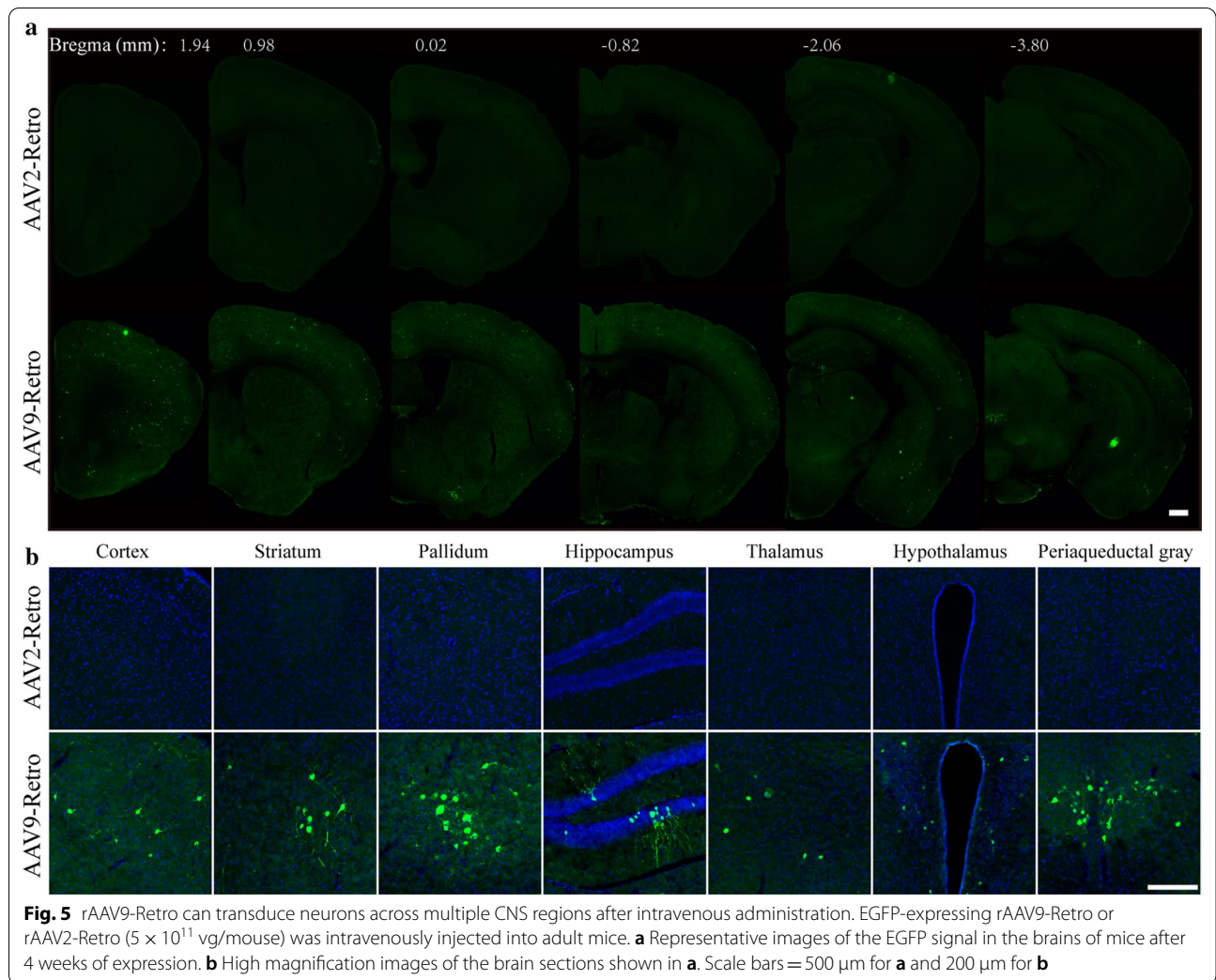


unknown. Therefore, it will be meaningful to compare the retrograde transduction efficiency between AAV9-Retro and AAV2-Retro in non-human primates, which may pave the path to gene therapy.

The treatment of many gene-deficient brain diseases, such as mucopolysaccharidosis IIIC, needs to compensate for the correct genes to every cell in the whole brain [44]. AAV, with effective retrograde infection or greater diffusion capacity, can achieve a wider range of drug delivery. AAV9 has a unique advantage over other serotype viruses—that is, it can transport across the

blood–brain barrier and target the whole brain by intravenous injection. The retrograde AAV based on AAV9 modification can also mediate efficient gene delivery to the brain by intravenous administration and be combined with local administration to achieve a better therapeutic effect, which will be addressed in a future study.

In summary, we have proven that AAV9-Retro can mediate efficient transduction with axon terminal absorption and blood–brain barrier transportation, providing an additional AAV vector tool for neuroscience and gene therapy.



Abbreviations

rAAV: Recombinant adeno-associated virus; CNS: Central nervous system; PRV: Pseudorabies virus; HSV: Herpes simplex virus; RABV: Rabies virus; LV: Lentivirus; CAV: Canine adenovirus; VTA: Ventral tegmental area; PFC: Prefrontal cortex; MO: Somatomotor areas; MSC: medial septal complex; MRN: midbrain reticular nucleus; ACA: anterior cingulate area; AI: agranular insular area; CPu: caudate putamen (striatum); CTX: cerebral cortex; BLA: basolateral amygdalar nucleus; TH: thalamus; DPI: days post-injection.

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Authors' contributions

KL and FX contributed to the study idea and design; FX contributed to funding acquisition and resources; XH contributed to the supply of adeno-associated viruses; KL, XZ, LL, MY and TY performed the experiments and data acquisition; KL, XZ, LL, ZZ and FX accomplished data analysis; KL and FX drafted the manuscript and contributed to reviewing and editing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All the procedures were approved by the Animal Care and Use Committees at the Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences. All the experiments with viruses were performed in a Biosafety Level 2 laboratory and animal facilities. Consent to participate: Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors here declare that there is no conflict of interest.

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