A Recombinant Baculovirus Efficiently Generates Recombinant Adeno-Associated Virus Vectors in Cultured Insect Cells and Larvae

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Current large-scale recombinant adeno-associated virus (rAAV) production systems based on the baculovirus expression vector (BEV) remain complicated and cost-intensive, and they lack versatility and flexibility. Here we present a novel recombinant baculovirus integrated with all packaging elements for the production of rAAV. To optimize BEV construction, ribosome leaky-scanning mechanism was used to express AAV Rep and Cap proteins downstream of the PH and P10 promoters in the pFast.Bac.Dual vector, respectively, and the rAAV genome was inserted between the two promoters. The yields of rAAV2, rAAV8, and rAAV9 derived from the BEV-infected Sf9 cells exceeded 10^5 vector genomes (VG) per cell. The BEV was shown to be stable and showed no apparent decrease of rAAV yield after at least four serial passages. The rAAVs derived from the new Bac system displayed high-quality and high-transduction activity. Additionally, rAAV2 could be efficiently generated from BEV-infected beet armyworm larvae at a per-larvae yield of 2.75 ± 1.66 × 10^10 VG. The rAAV2 derived from larvae showed a structure similar to the rAAV2 derived from HEK293 cells, and it also displayed high-transduction activity. In summary, the novel BEV is ideally suitable for large-scale rAAV production. Further, this study exploits a potential cost-efficient platform for rAAV production in insect larvae.

INTRODUCTION
Recombinant adeno-associated virus (rAAV) is a promising viral vector for gene transfer in both basic research and clinical gene therapy applications.1–5 In recent years, there has been an increasing need for rAAVs for large animal studies and human clinical trials.5–8 Many clinical trials using rAAV vectors have had positive outcomes such as in the treatments of monogenic disorders, including Leber congenital amaurosis,6 choroideremia,7 and hemophilia B.8 In 2012, the first rAAV-based drug Glycer was approved in the European Union for the treatment of lipoprotein lipase (LPL) deficiency.9 However, the very high cost of this drug means that many patients cannot afford access to it. As such, the cost-efficient large-scale production of rAAV, while keeping high yield and high quality, remains a major obstacle for rAAV clinical applications.10

The genome of the rAAV vector includes the exogenous gene of interest (GOI) flanked by inverted terminal repeats (ITRs), with the AAV Rep and Cap proteins being supplied in trans.11 Traditional plasmid transfection-based methods are difficult for scale-up production of rAAV, so alternatives must be considered.1,2,11 Although adenovirus (ADV) or herpes simplex virus (HSV) infection-based systems have developed for large-scale rAAV production, the downstream purification is hampered by existing pathogenic ADV or HSV.14–16 Among current large-scale rAAV production systems, the baculovirus expression vector (BEV)-mediated systems have unique advantages, such as high infection efficiency, the non-pathogenicity of BEV, and the cost-efficiency of serum-free Sf9 cell suspension culture.12,17 In 2002, Urabe et al. established the first-generation Bac system for the production of rAAV.18 In this system, Sf9 cells are co-infected with three BEVs: BEV/Rep, BEV/Cap, and BEV/(ITR-GOI). Because of the genetic instability of BEV/Rep and low co-infection ratio, this three-Bac system is not widely used.19

To improve the stability of BEV/Rep, two strategies have been developed to optimize the Rep expression cassette. In 2008, Chen developed a system to express AAV Rep and Cap proteins from a promoter within an artificial intron.20 In 2009, Smith et al. modified the AAV Rep gene to encode a single bi-functional mRNA transcript that could be translated into two distinct polypeptides, Rep78 and Rep52, by ribosome leaky scanning; meanwhile, the Cap gene was optimized to enhance VP1 protein translation through an optimal Kozak sequence and initiation codon.21 Thus, BEV/Cap-Rep was stable and showed no apparent loss of Cap and Rep protein expression.
even after five or seven consecutive amplifications. To overcome the drawback of low ratio with multi-BEV co-infection, Aslanidi et al. developed an inducible S9/Cap-Rep packaging cell line-based OneBac system in 2009. This strategy improves rAAV yield 10-fold compared with previously described Bac systems. In 2014, Mietzsch et al. used the OneBac system to produce the serotypes rAAV1 through rAAV12, and they showed that the rAAV yield reaches about 5 × 10^7 vector genomes per cell (VG/cell). Recently, Mietzsch et al. further developed an improved OneBac2.0 system, which can produce AAV5 with enhanced infectivity and AAV1, AAV2, AAV5, and AAV8 with minimal encapsidation of foreign DNA. However, even current packaging cell line-based OneBac systems still have some disadvantages, such as lack of versatility and flexibility, and it is difficult to obtain highly producing S9/Cap-Rep packaging cell line in the screening process.

In this study, we used an optimal strategy to integrate all rAAV-packaging elements, including the Rep gene, the Cap gene, and the rAAV genome (ITR-GOI), in a single BEV without disturbing one another's functions. We show that the yields of rAAV2, rAAV8, and rAAV9 derived from the new Bac system exceeded 10^5 VG/cell. We further show that the BEV/Cap-(ITR-GOI)-Rep is stable and showed no apparent decrease of rAAV yield after four serial amplification passages. We therefore posit that the new Bac system is versatile and flexible for large-scale rAAV production. Further, we demonstrate success in producing rAAV2 in beet armyworm larvae infected with the new BEV. These larvae-derived rAAV2 showed a structure similar to the rAAV2 derived from HEK293 cells and high-transduction activity, with an average rAAV2 yield exceeding 10^8 VG per larvae. The insect larvae could, therefore, be a potential cost-efficient platform for rAAV production.

RESULTS
Generation of rAAVs from Single Novel BEV-Infected S9 Cells
Current BEV-mediated production systems of rAAV have usually been based on S9 cells and recombinant baculovirus derived from Autographa california multiple nucleopolyhedrovirus (AcMNPV). In these Bac systems, the rAAV genome (ITR-GOI) is usually integrated in a single BEV, while the AAV Cap and Rep helper genes are supplied by other BEVs or S9 packaging cell lines. As of yet, there are no reports of a rAAV produced with a single BEV delivering all rAAV-packaging elements. As such, it is a huge challenge to combine all rAAV-packaging elements in a single BEV without functional disruptions. To achieve this goal, we have optimized the organization of the rAAV-packaging elements. The ribosome leaky-scanning mechanism was used to express AAV Rep and Cap proteins, as previously described. We chose the AccIII enzyme cut site to insert the rAAV genome (ITR-GOI) between the PH and P10 promoter regions of the pFast.Bac.Dual (pFD) plasmid. To facilitate rAAV detection, the rAAV genome used the cytomegalovirus (CMV) promoter-driven GFP reporter gene expression cassette flanked by AAV2 ITRs. Finally, we constructed the pFD/Cap-(ITR-GFP)-Rep shuttle vector (Figure 1A). Representative experiments used typical rAAV2 for functional validation. To obtain the BEVs, the pFD/Cap-(ITR-GFP)-Rep plasmids were transformed into the DH10Bac strain, and then the positive Bacmid DNA was transfected into S9 cells. By 72 hr post-transfection, the transfected S9 cells showed apparent GFP expression and cytopathic effect (Figure 1B). The data indicate that the BEV/Cap2-(ITR-GFP)-Rep was successfully generated. The culture supernatants of the transfected S9 cells were collected as BEV stock P1. We then used the P1 to infect naive S9 cells at an MOI of 3. After 72 hr post-infection, we collected the culture supernatants and harvested the cells.

The differences in thermo-stability between enveloped BEV and non-enveloped AAV allowed the specific inactivation of BEV by heat treatment at 60°C for 30 min. A simple infection-based method was used to test rAAV activity (Figure 1C). For rAAV2 (293 cell-derived) samples, in 293 cell-based infection assays, both the treated and untreated cells expressed GFP. In S9 cell-based infection assays, neither the treated nor untreated cells expressed GFP, indicating that rAAV2 did not infect S9 cells. For BEV/Cap2-(ITR-GFP)-Rep supernatant samples, which contained the major secreted BEV and some rAAV, in 293 cell-based infection assays, both the treated and untreated cells expressed GFP to some extent, with the GFP expression of the treated cells being significantly lower than that of the untreated cells. This was because inactive BEV did not express GFP and only some rAAV could express GFP. In S9 cell-based infection assays, the untreated cells expressed GFP, while the treated cells did not. For BEV/Cap2-(ITR-GFP)-Rep infected S9 cell lysate supernatant samples, which contained some non-secreted BEVs and the major rAAV, in 293 cell-based infection assays, both the treated and untreated cells expressed GFP, but GFP expression of the treated cells was slightly lower. This indicates that there was a large amount of rAAVs expressing GFP. In S9 cell-based infection assays, the untreated cells expressed GFP, while the treated cells did not express GFP (Figure 1C). To our surprise, the GFP expression level seemed very high under CMV promoter in the S9 cell. One reason may be the accumulation of GFP expression along with robust replication of the BEV. Another reason may be that unknown enhancer elements exist upstream of the CMV promoter in the BEV backbone. The results demonstrate that rAAV2 was successfully generated in the novel BEV-infected S9 cells.

The Productivity of the New Bac System
To determine the proper BEV infection ratio for rAAV production, the S9 cells cultured in a 6-well plate at 80% confluence were infected with BEV/Cap2-(ITR-GFP)-Rep at an MOI range from 0.1 to 30 with 3-fold dilutions. After 72 hr post-infection, the S9 cells were harvested, and the crude cell lysates were used to quantify the DNase-resistant rAAV particles. The yields of rAAV increased from 6.17 ± 1.15 × 10^4 VG/cell (MOI = 0.1) to 2.23 ± 0.41 × 10^5 VG/cell (MOI = 1), and they stably maintained a high level at MOIs from 1 to 30 (Figure 2A). The results indicate that the new Bac system exhibits high rAAV yield over a wide range of MOIs. As such, we chose an MOI of 3 for economical and practical considerations.

Further, we tested the yields of rAAV2, rAAV8, and rAAV9 derived from the new Bac system. The suspension-cultured S9 cells were
infected with the BEVs at an MOI of 3 (about 30 mL, 3 × 10^6 cells/mL). After 72 hr post-infection, the infected Sf9 cells were harvested and subject to iodixanol gradient purification. For rAAV2, rAAV8, and rAAV9, the yields were 2.24 ± 0.94 × 10^5 VG/cell, 3.23 ± 0.51 × 10^5 VG/cell, and 3.87 ± 0.83 × 10^5 VG/cell, respectively (Figure 2B). Taken together, the results demonstrate that the new Bac system is suitable for large-scale rAAV production.

Stability of BEV/Cap2-(ITR-GFP)-Rep upon Serial Passage

The new BEV/Cap2-(ITR-GFP)-Rep integrated all packaging elements for rAAV production, and so BEV stability is paramount for the scale-up production of rAAV. To this end, we amplified the BEV/Cap2-(ITR-GFP)-Rep for serial amplification passages from passage 1 to passage 8. For each passage, BEVs were allowed to infect naive Sf9 cells at an MOI of 0.1, and the culture supernatants were harvested as BEV stocks after 72 hr post-infection. Then, the Sf9 cells cultured in a 6-well plate at 80% confluence were infected with BEV/Cap2-(ITR-GFP)-Rep (MOI = 3) from passage 1 to passage 8. After 72 hr post-infection, the infected Sf9 cells were harvested. To determine whether the Rep and Cap coding sequences were stable in the baculoviruses, the passage samples were analyzed by western blot for Rep and Cap protein expression (Figure 3A). The expression of Rep78/Rep52 and VP1/VP2/VP3 proteins was clearly observed. Stable expression of the Rep and Cap proteins was observed to passage 4. From passage 5 to passage 8, both Rep and Cap expression decreased with passage number.

Because the yield level of rAAV could better reflect the BEV stability, we analyzed the yield level of rAAV upon consecutive passages of the BEV. The data show that the yields of rAAV maintained relatively
The demonstration of BEV stability level could, therefore, and maintains at least four serial passages without apparent decrease and Cap proteins. These results demonstrate that the BEV is stable and high levels and had no apparent decrease from passage 1 to passage 4 (all exceeding $10^5$ VG/cell). Beyond passage 4, the yields gradually decreased about 7-fold from passage 4 ($1.03 \pm 0.30 \times 10^6$ VG/cell) to passage 8 ($1.47 \pm 1.09 \times 10^6$ VG/cell) (Figure 3B). The yield level of rAAV correlated with the reduction of the expression level of Rep and Cap proteins. These results demonstrate that the BEV is stable and maintains at least four serial passages without apparent decrease in rAAV yield.

**Characterization of rAAVs Derived from Novel BEV-Infected Sf9 Cells**

To characterize the rAAVs produced by the new Bac system, iodixanol gradient-purified rAAV2, rAAV8, and rAAV9 were analyzed for their biophysical properties and transduction activity. The rAAV samples were denatured in loading buffer at 95°C for 5 min and then separated by 10% SDS-PAGE. After electrophoresis, gels were silver stained. The data show that the capsid protein compositions of rAAV2, rAAV8, and rAAV9 (Sf9 cell derived) were similar to rAAV2 (293 cell derived). The rAAVs displayed very high purity and presented three bands corresponding to the rAAV capsid proteins VP1 (87 kDa), VP2 (73 kDa), and VP3 (62 kDa), with a ratio of ~1:1:10 (Figure 4A). Transmission electron microscopy data showed that the virions of rAAV2, rAAV8, and rAAV9 (Sf9 cell derived) showed similar structures as rAAV2 (293 cell derived), as relatively uniform spherical particles with a diameter of 20–25 nm. As opposed to full particles, empty particles were distinguished on the basis of the electron-dense central region of the capsid. The data show that full particle contents of the rAAV2, rAAV8, and rAAV9 derived from the new Bac system were about 95.5%, 96.2%, and 94.9% in our preparation (Figure 4B).

Further, the transduction activities of the rAAVs were analyzed in the brains of C57 mice. The rAAV genome used carried a GFP reporter gene expression cassette driven by the CMV promoter. Similar to the rAAV2- (293 cell derived) injected mice, there was high GFP expression in the dentate gyrus (DG) near the injection sites in the rAAV2-, rAAV8-, and rAAV9- (Sf9 cell derived) injected mice. The fine structure of the GFP-labeled neuron could be seen clearly (Figure 4C). The results demonstrate that the rAAVs derived from the new Bac system exhibited high-quality and high-transduction activity in vivo.

**Generation of rAAVs from Novel BEV-Infected Insect Larvae**

In addition to insect cells, insect larvae have been widely used as a cost-efficient bioreactor for recombinant protein expression. Therefore, we sought to test whether rAAV could be generated from beet armyworm larvae upon infection of the novel BEV/Cap-(ITR-GFP)-Rep. After 3–4 days post-infection, the infected larvae showed apparent pathological symptoms, which were not observed in uninfected larvae (Figure 5B). We also observed remarkable GFP expression throughout the entire body of the infected larvae, but not in the uninfected larvae (Figure 5C). The GFP-expressing cells could be seen clearly in the subcutaneous tissue of the infected larvae, and they showed uneven distribution along the anteroposterior axis (Figure 5D). The results indicate that the larvae were efficiently infected with the novel BEV.

To confirm the production of rAAV2 in the BEV-infected larvae, we used cell-based infection assays to test the rAAV activity as previously described in Figure 1C. For the crude uninfected larvae lysate supernatant samples, in both 293 and Sf9 cell-based infection assays there was no GFP expression (Figure 6A). For the crude infected larvae lysate supernatant samples, in 293 cell-based infection assays, both the treated and untreated cells expressed GFP, but GFP expression of the treated cells was slightly lower. It indicates that there were large amounts of rAAV expressing GFP. In Sf9 cell-based infection assays, untreated cells expressed GFP, but the treated did not (Figure 6A). The results indicate that rAAV2 was successfully produced in the BEV-infected larvae.

To test the infectivity of the rAAV derived from larvae, we compared the transduction activity of rAAV2 derived from HEK293 cells, Sf9 cells, and larvae infected in cultured HEK293 cells. The gradient diluent rAAV2 was infected with HEK293 cells, and GFP fluorescence was detected 2 days post-infection. The result indicates that this rAAV2 exhibited similar transduction activities. The purified rAAV2 derived from larvae was then analyzed by transmission electron microscopy. The data show that rAAV2 virions were present as relatively uniform spherical particles with a diameter of 20–25 nm (Figure 6C). This result from larvae-derived rAAV2 is consistent with the transmission electron microscopy (TEM) result from HEK293 cell-derived rAAV2 (Figure 4B). The purified rAAV2 ($\sim 2.0 \times 10^6$ VG) derived from
larvae was then injected into the mouse brain DG. The data show that there was high GFP expression in the DG and the fine structure of GFP-labeled neurons could be seen clearly (Figure 6D). These results demonstrate that the rAAVs derived from larvae have high-transduction activity in vivo.

Next, we assessed the rAAV2 yield in the novel BEV-infected beet armyworm larvae. The data showed that the average rAAV2 yield per larva was $2.75 \pm 1.66 \times 10^{10}$ VG in our preparation. Further, we analyzed the rAAV2 yield in different parts of the larvae. The data showed that there were about $0.78 \pm 0.12 \times 10^{10}$ VG, $0.39 \pm 0.14 \times 10^{10}$ VG, and $1.91 \pm 0.19 \times 10^{10}$ VG in the hemolymph (25.30%), gut (12.57%), and other tissues (62.13%), respectively (Figure 6E). These results suggest that the larvae are a cost-efficient platform for rAAV production.

**DISCUSSION**

In 2002, Urabe et al. demonstrated that the rAAV vector could reproduce in cultured S9 cells upon triple BEV infection. The results suggest that insect cells support the replication of AAV, and baculovirus infection could supply the helper function, although the detailed mechanism is not known. Since then, further improvements have been made in two key aspects: (1) to improve genetic stability of the BEVs, and (2) to reduce the complexity of the Bac system. In short, AAV Cap and Rep helper genes were incorporated into a single BEV or integrated into S9 packaging cell lines, and the two-Bac systems and S9 packaging cell line-based OneBac systems were generated. However, current Bac systems still remain complicated and cost-intensive, and they lack versatility and flexibility.

The ideal solution is developing an efficient BEV integrating all the necessary packaging elements for rAAV production. As such, it is a difficult challenge to construct a BEV to such specifications. One difficulty is how to organize the Cap and Rep genes as well as the rAAV genome (ITR-GOI) in a single BEV while ensuring that the organization does not disrupt another’s functions. Another main concern is genetic stability of the highly integrated BEV. Moreover, the AAV ITR sequences are susceptible to AAV Rep-mediated cleavage. Whether the progeny BEV remain integrated in the situation of the simultaneous existence of the expression of the Rep gene and rAAV packaging is also of concern. Thus, the designs of the BEV constructs should consider two rules. First, the Rep and Cap genes and (ITR-GOI) must be optimally organized without disrupting one another's functions. Second, the constructs should be versatile and flexible for different kinds of rAAV production. To this end, we integrated them into a single pFD shuttle vector, thus facilitating BEV construction through Bac-to-Bac operation. Based on previous achievements of the Bac systems, we employed the ribosome leaky-scanning mechanism to express AAV Rep and Cap proteins downstream the PH and P10 promoters in the pFD vector, respectively. In the pFD vector, the PH and P10 promoters are head-to-head linked by a short DNA fragment, which contains an AcclI cut site. This site is suitable for the insertion of the (ITR-GOI). To minimize the adverse impact of insertion, we brought in short linker DNA fragments (about 80–140 bp) outside of each ITR through PCR amplification. Finally, the functional BEV/Cap-(ITR-GFP)-Rep was produced from the Bac-mid-transfected S9 cells (Figures 1A and 1B). The rAAVs were successfully generated from the S9 cells upon BEV/Cap-(ITR-GFP)-Rep infection (Figure 1C).

The new Bac system displayed a wide range of MOIs for high-level rAAV production (Figure 3A). This property makes it suitable for optimizing the fermentation process at high cell density. The yield levels of rAAV2, rAAV8, and rAAV9 derived from the new Bac systems exceeded $10^8$ VG/cell (Figure 3B), which is close to the current best states of large-scale rAAV production systems. To our delight, the BEV/Cap-(ITR-GFP)-Rep was shown to be stable over at least four amplification passages. Moreover, the expression of Rep and Cap proteins maintained stable levels during passages 1–4 and gradually decreased from passages 5–8, which is correlated with the yield levels of rAAV (Figure 4). Compared with the previously reported BEV/Cap-Rep, which showed stable expression of the AAV Rep and Cap proteins over seven passages, the apparently lower stability of BEV/Cap-(ITR-GFP)-Rep could due to the additional ITR-containing component, as there was a notable loss of the ITR-transgene cassette-containing baculovirus over the five passages, as previously reported. Further improvements to the stability of the new BEV are needed, and a recent study on lef5 should be noted in
In addition to cultured insect cells, the insect larvae have been widely used for exogenous protein expression. Although rAAV has been successfully produced in cultured S9 cells for years, there are as yet no reports that rAAV has been produced in insect larvae. This is because, unlike in cultured S9 cells, it is hard to control multi-BEV co-infection with insect larvae. Also, unlike in the stable S9 packaging cell line, it is very difficult to gain transgenic insects suitable for the production of rAAVs. The successful production of functional BEV/Cap-(ITR-GFP)-Rep, therefore, paves the way for efficient production of rAAV in insect larvae. A further consideration is that the best armyworm is the natural host of the AcMNPV-derived BEV/Cap-(ITR-GFP)-Rep in this study. In the present study, we tested whether rAAV could be produced in the BEV/Cap-(ITR-GFP)-Rep-infected beetle armyworm larvae. We found that the BEV replicated efficiently throughout the entire larval body after 3–4 days of intrahemocoelical injection (Figure 5). The rAAV2 was successfully generated in BEV-infected beetle armyworm larvae (Figure 6A). The larvae-derived rAAV2 show similar in vitro and in vivo transduction activity to the HEK293- and S9-derived rAAVs (Figures 6B and 6D). The average yield of rAAV2 per larvae was observed to be 2.75 ± 1.66 × 10^{10} VG in our preparation. Unlike secreted proteins, which mainly exist in the larvae hemolymph, about 74.7% of the rAAV existed in the larvae tissues (Figure 6E). An efficient purification method of rAAVs produced in insect larvae still needs to be further developed. Compared to intrahemocoelical injection, oral infection is more suitable for scale-up production of rAAV in insect larvae due to less labor-intensive manual operation. Thus, oral infection might be better for large-scale production of rAAV in insect larvae. Moreover, to find the ideal insect larvae bioreactor for the production of rAAV, it will be worth testing the rAAV productivity with different types of recombinant baculovirus and insect larvae in the future, such as recombinant Bombyx mori nucleopolyhedrovirus (BmNPV) and its host silkworm (Bombyx mori) larvae, which have been widely exploited.

In conclusion, high-quality rAAVs were produced in the new BEV/Cap-(ITR-GFP)-Rep-infected S9 cells. The yields of rAAV2, rAAV8, and rAAV9 exceeded 10^5 VG/cell, which is close to the current best states of large-scale rAAV production systems. The new BEV is stable for at least four amplification passages. The new Bac system is ideally suitable for large-scale rAAV production. In addition, it is the first demonstration of rAAV2 production in beetle armyworm larvae using the new BEV infection. The rAAV2 derived from larvae show a structure similar to the HEK293 cell-derived rAAV2 and high-transduction activity, both in cultured HEK293 cells and mouse brain DG. The average rAAV2 yield exceeded 10^{10} VG per larvae, and the rAAV2 was seen to mainly exist in larval tissues. Thus, this study exploits a potential cost-efficient platform for rAAV production in insect larvae.

**MATERIALS AND METHODS**

**Plasmid Construction**

An artificially synthesized AAV2 Rep gene expressing a bifunctional Rep78- and Rep52-encoding mRNA was amplified with primers Rep-Bgl-F and Rep-Xba-R, cloned into pFD plasmid between BamH1 and Xba1 cut sites to create the pFD/Rep. The Cap2, Cap8, and Cap9 genes were amplified from pAAV-Rc2, pAAV-Rc8, and pAAV-Rc9 with primers Cap2-Xma-F and Cap2-Nhe-R, Cap8-Xma-F and Cap8-Nhe-R, and Cap9-Xma-F and Cap9-Nhe-R, and then they were cloned into pFD between Xma1 and Nhe1 cut sites to create the pFD/Cap2, pFD/Cap8, and pFD/Cap9, respectively. 

The ITR-(L) and ITR-(R) fragments were amplified from pAAV-MCS.
with primers ITR-KpnM-F and Sac1-R or primers Kpn-Sac1-F and ITR-SpeM-R, and then they were ligated into psimp1T-ITR. A GFP reporter gene was amplified by primers EcoR-GFP-F and GFP-BamH-R, and then it was inserted into psimp1T-ITR between EcoR1 and BamH1 cut sites to create psimp1T-(ITR-GFP). The (ITR-GFP) fragment was inserted into pFD/Cap2 between Mro1 and Spe1 cut sites to create pFD/Cap2-(ITR-GFP). The PH-Rep fragment was amplified from pFD/Rep with primers Rep-Spe-F and RepXba-R, and it was inserted into pFD/Cap2-(ITR-GFP) at the Spe1 cut site to create pFD/Cap2-(ITR-GFP)-Rep. The Cap2 gene in pFD/Cap2-(ITR-GFP)-Rep was replaced by Cap8 or Cap9 genes from pFD/Cap8 or pFD/Cap9 through Pac1 and Nhe1 cut sites to obtain pFD/Cap8-(ITR-GFP)-Rep or pFD/Cap9-(ITR-GFP)-Rep, respectively. Recombinant baculoviruses were generated following the Bac-to-Bac system protocol (Invitrogen). All constructs were confirmed by sequencing. Primers used for PCR are shown in Table S1.

Cell and Insect Larvae Culture
HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Adherent culture of Sf9 cells was maintained in plate cultures containing Grace’s Insect Medium (Gibco) supplemented with 10% FBS, at 28°C. Suspension culture of Sf9 cells was maintained in shake flask cultures containing SF-900II SFM (Gibco), at 28°C. Media were supplemented with 100 μg/mL streptomycin and 100 U/mL penicillin. The beet armyworm (Spodoptera exigua) larvae were raised at 25°C under 24-hr:10-hr light:dark cycle with an artificial diet.

Larvae Sample Preparation and Observation
For beet armyworm larvae injection, the fifth-instar larvae were injected by intrahemocelical injection with 10 μL (~1.0 × 10⁵ VG) cold ice, and then they were surgically dissected to collect the hemolymph, gut, and other tissues (mainly including the fat body and epidermis). The larvae or tissue samples were then homogenized in PBS buffer, and then they were subjected to three freeze-thaw cycles with liquid nitrogen and a 37°C water bath, followed by centrifugation at 5,000 × g for 10 min. After centrifugation, the larvae or the tissue crude lysate supernatants were immediately subjected to downstream operation or stored at −80°C until use. The rest of the extraction procedure for the larvac-derived rAAV was similar to that used for Sf9 cell-derived rAAV. For the observation of larvae, the larvae were narcotized on cold ice, and whole-larvae fluorescence imaging was carried out by an in vivo imaging system (CRI Maestro2). Low-magnification imaging was carried out by stereo microscope (Olympus SZX16), and high-magnification imaging was carried out by inverted microscope (Olympus IX71).

rAAV Production and Purification
rAAV production in HEK293 cells was performed as previously described. Briefly, HEK293 cells at 80% confluence were co-transfected with polyethylenimine (PEI) method with plasmids pAAV-RC2, pAAV-helper, and pAAV-GFP. Cells were harvested 3 days post-transfection. For Sf9 cells, adherent cultured insect cells at 80% confluence were infected with BEV at an MOI of 3. Suspension-cultured insect cells at 3 × 10⁶ cells/mL density were infected with BEV (MOI = 3). Cells were harvested 3 days post-infection. HEK293 cells or Sf9 cells were suspended in lysis buffer (50 mM Tris and 2 mM MgCl₂ [pH 7.5]) at a cell density of 2 × 10⁷ cells/mL. Cells were lysed by three freeze-thaw cycles using liquid nitrogen and a 37°C water bath. The crude cells lysates were mixed with 50 U/mL benzonase (Sigma) with the addition of 150 mM NaCl for 1 hr at 37°C. Then,
the mixture was centrifuged at 2,500 × g for 15 min and filtered. The supernatant fractions were used for downstream purification.

An iodixanol step density gradient was prepared as described previously. Briefly, 15%, 25%, 40%, and 58% iodixanol were diluted in PBS-MK Buffer (1× PBS, 1 mM MgCl₂, and 2.5 mM KCl) from 60% iodixanol (OptiPrep; Sigma). The cell lysate samples and iodixanol solution were successively underlaid in 39 mL Quick-Seal tubes (Beckman Coulter). After centrifugation in a 70 Ti rotor for 2 hr at 63,000 rpm and 18°C, the fractions obtained from the 40% phase were dialyzed against PBS and then ultrafiltered and concentrated, using Amicon Ultra-15 centrifugal filter units (MWCO, 100 kDa; Merck Millipore). The purified rAAVs were aliquoted and stored at −80°C.

**Virus Titration**

Virus titers were determined by qPCR assay with the iQ SYBR Green Supermix kit (Bio-Rad). A standard curve was obtained using 10-fold serial dilutions of plasmids. The BEV samples were treated with a modified alkaline polyethylene glycol (PEG)-based method as previously described. The preparations with highly purified rAAV vector or rAAV in crude lysate supernatants were performed as previously described. For the titration of the rAAV derived from crude lysates of Sf9 cell, the crude lysates were heated to inactivate the BEV before benzonase treatment.

Figure 6. Analysis of rAAV Produced in Beet Armyworm Larvae upon the New BEV/Cap2-(ITR-GFP)-Rep Infection

(A) Fluorescence microscopy observation of HEK293 and Sf9 cells infected with the supernatant of infected or uninfected larval lysate samples. Treated, heat treatment with 60°C for 30 min; p.i., post-infection. Representative fields are shown; upper images are green fluorescence fields, lower images are bright fields. (B) Comparison of transduction efficiency among rAAV derived from HEK293 cells, Sf9 cells, and larvae. HEK293 cells were infected with gradient diluents of rAAVs and examined under a fluorescence microscope 2 days post-infection. (C) Transmission electron microscopy analysis of negatively stained purified rAAV2. Original magnification ×100,000. Scale bars, 100 nm. (D) Transduction activity analysis of larvae-derived rAAV2 in mouse brain DG. Scale bar, 500 μm. Representative fields are shown. (E) Analysis of the yields of rAAV2 in different parts of the larvae. All experiments were done in triplicate and displayed as mean ± SD.
The quantitative primers for BEV and rAAV were Bac-F and Bac-R and CMV-F and CMV-R, respectively. Primers used for qPCR are shown in Table S1.

**Western Blot and Silver Staining**  
Western blot analysis was performed as previously described. Samples were separated by using 10% SDS-PAGE. Rep proteins were detected using an anti-Rep monoclonal antibody (clone 303.9, Fitzgerald) at a 1:100 dilution. A monoclonal antibody against β-tubulin (Proteintech) was used at a 1:1,000 dilution. Secondary detection antibodies were used at a 1:2,000 dilution. For silver staining, fixed gels were stained using the Fast Silver Stain Kit (Beyotime) according to the manufacturer’s protocol.

**Transmission Electron Microscopy**  
The virus particles were assessed by negative stain and transmission electron microscopy. An approximately 10-μL sample was placed on a 400-mesh carbon-coated copper grid and incubated for 2 min, quickly removed, and air dried. The grid was then washed with 2% phosphotungstic acid (pH 7.4) for 2 min, washed with a drop of water, and then air dried again. Experiments were performed with a transmission electron microscope (Hitachi H-7000A).

**rAAV In Vivo Property Assay**  
All animal experiment protocols were approved by Animal Care and Use Committees at the Wuhan Institute of Physics and Mathematics, the Chinese Academy of Sciences. 8-week-old male C57BL/6 mice were used for the virus transduction assay. Briefly, the mice were anesthetized with chloral hydrate and secured in a stereotaxic apparatus. The skull of the targeted site was thinned by a dental drill. A syringe (10 μL) connected to a glass micropipette with a 10- to 15-μm diameter tip was used for virus injection. The rAAV (~2.0 × 10^8 VG) in a volume of 0.2 μL was stereotactically injected into the DG, using the coordinates 1.70 mm posterior to bregma, 1.00 mm lateral from midline, and 2.0 mm dorsoventral relative to bregma. The syringe was left in place for 10 min following injection to minimize diffusion. At 3 weeks after injection, the mice were sacrificed. Mice were deeply anesthetized with an overdose of chloral hydrate and transcardially perfused with 0.9% saline solution followed by 4% paraformaldehyde (PFA) solution. The brains were removed, then fixed in 4% PFA overnight, and dehydrated in 30% sucrose before being sectioned into 40-μm slices (Thermo Fisher Scientific). Fixed slices were stained with DAPI and imaged by virtual slide microscope (Olympus VS120).

**SUPPLEMENTAL INFORMATION**  
Supplemental Information includes one table and can be found with this article online at https://doi.org/10.1016/j.omtm.2018.05.005.

**AUTHOR CONTRIBUTIONS**  
Y.W. conceived and performed experiments, analyzed the data, and wrote and edited the manuscript. L.J. performed experiments and analyzed the data. H.G. and Z.H. carried out viral vector production. T.Y. carried out animal studies. X.H. contributed to the conception and design of experiments. K.L. contributed to larval experiments. F.X. designed experiments, supervised the project, and wrote and edited the manuscript.

**CONFLICTS OF INTEREST**  
The authors declare no competing financial interests.

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