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ORIGINAL ARTICLE

Intravenous injection of AAV-PHP.eB across the blood-brain barrier in the adult mouse for central nervous system gene therapy*

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Abstract

Objective To verify the neurotypicality of AAV-PHP.eB after tail vein injection in adult mice and its efficiency in crossing the blood-brain barrier (BBB).

Methods The rAAV-SYN-GFP plasmid was constructed, and adult C57BL mice were injected with AAV-PHP.eB: SYN-GFP in the tail vein (300 nL, virus titer 3×10^9 vg) and in the prefrontal lobe (50 L, virus titer 5×10^{11} vg). The green fluorescent protein (GFP) signal in the brain was observed at two weeks, while the GFP signal in the peripheral organs was observed at four weeks.

Results Two weeks after tail vein injection, GFP expression was observed throughout the brain, especially in the cortex, hippocampus, and geniculate nucleus. No GFP signal was observed or detected by western blotting in the peripheral organs after four weeks. GFP signal was observed mainly at the local site after prefrontal lobe injection.

Conclusion AAV-PHP.eB: SYN-GFP can effectively cross the BBB in adult mice. Using a neuron-specific promoter allows exogenous gene expression in neurons; therefore, AAV-PHP.eB can be used as an effective carrier for studying diseases in the central nervous system (CNS).

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Key words: gene therapy; AAV-PHP.eB; blood-brain barrier; regulatory element; noninvasive viral injection

Over the past 20 years, gene therapy has been developed greatly [1]. In particular, recombinant adeno-associated Viruses (rAAV) are commonly used for delivering genes and vaccines. Safety and efficacy have been evaluated in the clinical trials of therapies for different diseases, including Leber's congenital amaurosis type 2, Duchenne muscular dystrophy, and hemophilia B [2–5].

AAVs are a kind of non-enveloped single-stranded DNA virus classified as a parvovirus, and their replication depends on the presence of adenoviruses or herpes simplex viruses (also called helper viruses) [6]. They can establish a latent infection in different cell types while having no association with any human disease, making them potential vectors for carrying exogenous transgenes. With genetic engineering, genomes as big as 4.7 kilobases can be loaded and inserted in a region flanked by two inverted terminal repeats, rep for AAV replication and cap for structure proteins and helper genes, and therapeutic rAAV can be produced.

Variant AAV capsid sequences generate different serotypes, and currently the 11 most common AAV variants (AAV1–AAV11) have been elucidated from human and non-human tissues [7]. Most serotypes show neuron tropism, including AAV1, AAV2, AAV5, and AAV9 [8–10]. These neurotropic viral vectors are promising candidates for the treatment of central nervous system

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(CNS) diseases.

With increasing interests in gene therapy for CNS diseases, more basic research and pre-clinical trials are currently being conducted. A key issue is finding efficient vector delivery strategies to overcome limited rAAV transduction efficiency due to the blood-brain barrier (BBB). Though direct intracranial injection is effective for diseases with a localized lesion such as Parkinson's disease, other diseases such as glioblastoma multiforme (GBM), lysosomal storage diseases (LSDs), Alzheimer's disease, and Canavan's disease require widespread vector delivery to systematically manipulate pathological changes [11–14]. Under these circumstances, a better approach is to administer the vectors through vasculature injections for global delivery [15]. In addition, in contrast to intracranial injections that can cause brain injury, relatively noninvasive intravenous or intra-arterial injections can lower surgical risks.

Among the mentioned neurotropic characteristic of AAV serotype variants, AAV9 is the only serotype that can efficiently get through the BBB after intravenous injection in adult murine models [16], which makes it practical for further studies on noninvasive viral injection targeting the CNS [17]. According to a review in 2017, AAV-PHP.eB has been applied in clinical trials on neurodegenerative disorders including Parkinson's disease [19–20] and Alzheimer's disease [15].

Besides neurotropic serotypes, strong expression promoters like cytomegalovirus (CMV), CMV early enhancer/chicken β-actin (CAG), and phosphoglycerate kinase promoters are also considered and are the most widely used in AAV vectors for robust transgene expression, both in neurons and non-neuronal cells such as motor neurons, astrocytes, and Purkinje cells [21, 16]. Different neurons in the CNS form circuits involved in relative independent functions; thus, how to target a gene to a particular area and how it performs under expected conditions are big challenges for AAV clinical application, driving us to carefully consider expression patterns after AAV transduction. Cell type-specific transgene expression can provide a more tailored expression pattern by targeting certain cell types during pathogenesis. Human synapsin (hSyn) is a commonly used promoter to limit expression in neurons [22].

According to an updated report, AAV-PHP.eB performs as the most efficient serotype that can cross the BBB and rapidly diffuse in the CNS after intravenous administration. We want to establish a noninvasive delivery strategy with a high transduction efficiency and tissue specificity. Here, we report that, by applying the Syn promoter with the regulatory element WPRE that can improve GFP expression, we observed high neuronal specificity of expression and high efficiency of delivery of the exogenous gene in the nervous system, demonstrating the tissue specificity and the potential for wider usage of this gene delivery strategy.

Materials and Methods

Animals

C57BL mice were bought from Shanghai Jiesijie Laboratory Animal Technology Company.

Vector design and virus production

Vector information containing the hSyn promoter was obtained on Addgene (No.50465), and the gene vector hSyn-EGFP is as follows (Fig.1).

Plasmid AAV-PHP.eB was purchased from PackGene Biotech, LLC. The vector genome, the helper plasmid containing cap9 sequences, and adenovirus genes were transfected into 293 cells, and the virus was harvested from lytic 293 cells and culture supernatants. A dose of 1×10^{13} vector genomes (vg) virus was obtained for animal injection.

Virus administration

Virus injections were carried out in 8-week-old male C57BL mice with body weight of 250–300 g. In order to find the vessel, two white mice were injected in the tail vein, while the virus was delivered through the prefrontal lobe in two black mice assisted with a brain stereotaxic apparatus (Olympus, Japan).

Tissue preparation

At two and at four weeks after administration, one mouse of each injection route was obtained for cardiac perfusion. Phosphate-buffered saline (PBS) was kept running, followed by treatment with 4% paraformaldehyde (wt/vol) until the limbs of the mouse became stiff. Then, the brain and the internal organs, including the heart, liver, kidney, and lungs, were immersed in 4% paraformaldehyde and stored at 4 °C overnight. After dehydration by 15% and 30% (wt/vol) sucrose, tissues were embedded in Optimal Cutting Temperature compound and frozen at –80 °C for cryostat sectioning (Thermo).

Immunohistochemistry

Tissue slices (50 μm) were obtained for fluorescent staining. Briefly, slides were incubated at room temperature and washed with 10 μM PBS (pH 7.4) three times before blocking with 5% bovine serum albumin (BSA) and 0.3% Triton X-100 in 10 μM PBS for two hours. After washing with PBS three times, the slices were incubated with primary rabbit-derived GFP antibody (1:1000; Vector, USA) overnight at 4℃. Slices were subsequently washed with PBS, incubated with fluorescent dye labeled with donkey anti-rabbit IgG (488,

1:1000; Alexa Fluor, Invitrogen) for 30 mins, and then stained with DAPI (1:400; Haoran Biotech, Shanghai) for 20 mins to label the nuclei. After washing with PBS, the slides were mounted with non-fluorescent mounting media (Hydromount). Images were captured through a confocal microscope (Nikon-TIE-A1, Japan).

Western blotting

Tissue sections obtained from the brain, heart, kidney, lung, and stomach of intravenously injected mice were homogenized for western blotting. Primary antibodies of mouse anti-GFP (1:3000; Abcam, USA) and mouse anti-actin (1:5000; ProteinTech, USA) were added. Peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000; Yeasen, China) was used. Protein bands were visualized by western blotting substrate (Tanon, China) and imaged using the chemiluminescence instrument (Tanon, China).

Results

First, we constructed a plasmid in which the AAV-PHP.eB sequence mediated the expression of EGFP under the control of the Syn promoter (Fig. 1). Then, virus was harvested from 293 cells and supernatants. Finally, we got 1×10^{13} vector genomes (vg) high-titer viral preparations.

In order to compare the transduction efficacy of different routes of administration, we separately employed tail vein injection and intracranial injection in 8-week-old non-transgenic mice (C57BL), as demonstrated in Fig. 2a. Two weeks after tail vein AAV-SYN-GFP administration, the global-scale nervous system was labeled by GFP fluorescence (Fig. 2b). GFP was particularly and strongly expressed in the cerebral cortex, hippocampus, geniculate nucleus, and cerebellar Purkinje fibers (Fig. 2d, 2e). Strong GFP fluorescence was also observed near the area of vector delivery two weeks after intracranial injection (Fig. 2c); beyond that area, GFP signal was sparsely observed. These results showed the global expression pattern of the target gene delivered by tail vain administration and that its efficacy is greater than that by prefrontal lobe injection. As previously described, we also further confirmed that these vectors can cross the BBB.

As GFP was driven by the neuron-specific promoter Syn, we clarified the GFP fluorescence in the internal organs four weeks after intravenous delivery. Though the background signal was high, we found scarce punctiform GFP signals in other tissues, including the kidney, lung, heart, stomach, and liver (Fig. 3). The result demonstrated high CNS specificity using AAV-PHP.eB with a neuron-specific promoter. In the liver, we observed different sectioned areas with high fluorescent background, but observed limited GFP signal. In order to further clarify the neurotropic characteristic of AAV vectors, tissues from different organs, including the midbrain, heart, kidney, lung, and stomach, were homogenized for western blotting (Fig. 4). Results further confirmed that GFP was only expressed in the brain, demonstrating the tissue-specific expression pattern of AAV vectors.

Discussion

Here, we demonstrated the capability of the new AAV capsid AAV-PHP.eB vector for specific targeting driven by a specific promoter with a substantial dose. We clarified that AAV-PHP.eB has two important advantages for application in gene therapy: First, the low required

Fig. 1 Schematic view of gene vector

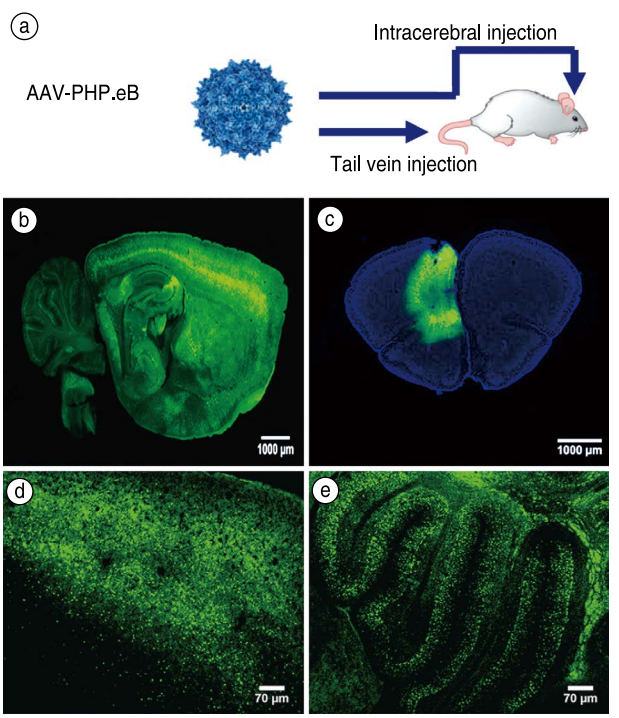

Fig. 2 Global GFP expression in the central nervous system after AAV-PHP.eB: SYN-GFP intravenous injection. (a) Schematic view depicting the operation of AAV-PHP.eB injection into the tail vein and the prefrontal lobe of an adult C57BL mouse; (b) After virus injection through the tail vein at 5×10^{11} vg per viral vector, the whole brain area showed strong GFP expression, especially in the cortex, hippocampus, and geniculate nucleus; (c) The virus was directly injected into the prefrontal lobe at 3×10^{9} vg per viral vector. The GFP fluorescence of the entire brain tissue was mainly concentrated around the injection site; (d) GFP-positive local area of cerebral cortex via tail vein injection; (e) GFP-positive local area of cerebellar Purkinje fibers

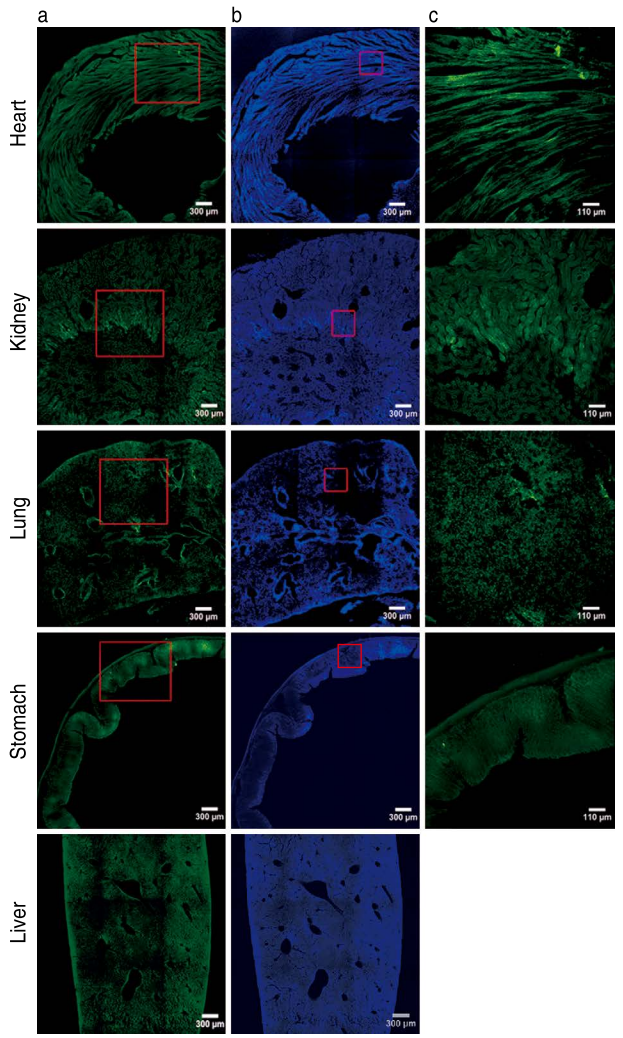

Fig. 3 No GFP signal was observed in the internal organs with AAV-PHP.eB: SYN-GFP intravenous injection after 4 weeks. Images show fluorescence expression by 2 different channels. Representative images of GFP channel (column a, green) and DAPI channel (column b, blue). (c) Amplified tissue images in red frame of column a. Scale bars were 300 μm (a, b) and 110 μm (c)

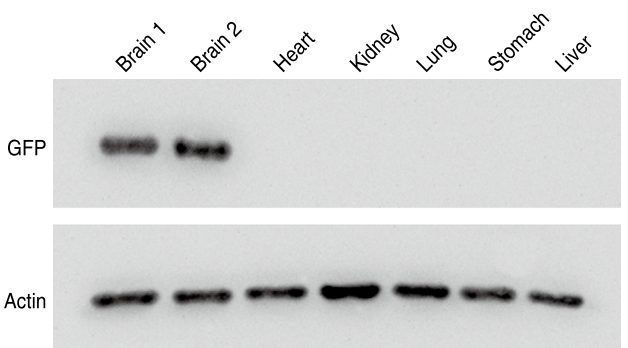

Fig. 4 No GFP expression in the internal organ tissues with AAV-PHP.eB: SYN-GFP intravenous injection after 4 weeks. The results further confirmed that GFP was expressed only in midbrain tissues

viral dose of AAV-PHP.eB (~10^{11} vg) that can cross the BBB and achieve effective expression in the target area via a tissue-specific promoter. Second, intravenous administration of AAV-PHP.eB: SYN showed little off-target effects on the liver, lung, and other peripheral organs.

It was previously reported that, after intravenous injection of AAV9, infected cells can be found in the CNS and in the peripheral nervous system (PNS) in neonatal mice, and in astrocytes and some hippocampal neurons during adulthood [23–24]. This initiated the development of various noninvasive viral delivery systems for the treatment of diseases of the CNS. To date, noninvasive gene delivery systems targeting the CNS have been widely increasing. It was reported that three fluorescent proteins were separately packed into AAV-PHP.eB with hSyn1 promoter and mixed vectors showed the multicolor hues in the cortex, striatum, and cerebellum after intravenous administration in non-transgenic mice. This indicated efficient transduction, and that AAV-PHP.eB with a neuron-specific promoter can be a promising tool for gene delivery to specific cell types in the brain. More interestingly, another type of AAV-PHP.S capsid was also reported to specifically transduce dorsal root ganglia, as well as cardiac and enteric neurons [25].

For viral gene therapy targeting CNS diseases in the future, it should be carefully considered if the goal is to express the target gene in as many cells systemically, or to express the gene in target cells of a local region. In the brain, rAAV9 delivery can initially transduce neurons and astrocytes using a strong CBA promoter, though the ratios varied across studies [23, 26].

Our experiment used the most common neuron-specific promoter hSyn. We clearly observed GFP expression in many regions, including the cortex and hippocampus, the two most studied areas for brain function. We observed a wider GFP expression pattern in the substantia nigra pars reticulata, motor cortex, and hippocampus by neonatal intracerebroventricular injection of rAAV9 [27], which is consistent with our observation, demonstrating that intravascular injection in adult mice also shows efficient transduction results like that in neonatal mice. Since rAAV9 reduces neurotropism and favors astroglial transduction as mice grow up, using a cell type-specific promoter can avoid this change; however, how the transduction efficiency is affected at different stages still requires further study. In addition, whether engineered AAV-PHP.eB shows a different transduction efficiency compared with that of rAAV9 also requires further clarification. All these studies are critical for determining the best injection window and load in treating long-term progressive diseases.

In order to study the function of genes in different brain diseases, as well as to find therapeutic targets, more

tissue-specific promoters like CamkIIα for excitatory neurons and strong promoters like CMV and CAG should be tested [28–29]. This will promote further studies and applications of AAV gene therapy in CNS diseases in the future.

No current clinical treatments can prevent the progression of CNS diseases; however, gene therapy has a strong potential to "permanently" cure disease by replacing a dysfunctional copy of a gene with a normal one [24]. Though many challenges from basic research to technical application remain to be addressed, clinical gene delivery trials with AAV-PHP.eB are increasing, and we believe that these will push viral gene therapy forward.

Conflicts of interest

The authors declare no potential conflicts of interest.

References

1. Saraiva J, Nobre RJ, Pereira de Almeida L. Gene therapy for the CNS using AAVs: The impact of systemic delivery by AAV9. J Control Release, 2016, 241: 94–109.
2. Bainbridge JW, Mehat MS, Sundaram V, *et al*. Long-term effect of gene therapy on Leber's congenital amaurosis. N Engl J Med, 2015, 372: 1887–1897.
3. Cideciyan AV, Hauswirth WW, Aleman TS, *et al*. Human RPE65 gene therapy for Leber congenital amaurosis: persistence of early visual improvements and safety at 1 year. Hum Gene Ther, 2009, 20: 999–1004.
4. Nathwani AC, Tuddenham EG, Rangarajan S, *et al*. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med, 2011, 365: 2357–2365.
5. Testa F, Maguire AM, Rossi S, *et al*. Three-year follow-up after unilateral subretinal delivery of adeno-associated virus in patients with Leber congenital Amaurosis type 2. Ophthalmology, 2013, 120: 1283–1291.
6. Schaffer DV, Koerber JT, Lim KI. Molecular engineering of viral gene delivery vehicles. Annu Rev Biomed Eng, 2008, 10: 169–194.
7. Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. Mol Ther, 2006, 14: 316–327.
8. Bartlett JS, Samulski RJ, McCown TJ. Selective and rapid uptake of adeno-associated virus type 2 in brain. Hum Gene Ther, 1998, 9: 1181–1186.
9. Mandel RJ, Burger C. Clinical trials in neurological disorders using AAV vectors: promises and challenges. Curr Opin Mol Ther, 2004, 6: 482–490.
10. Cearley CN, Wolfe JH. A single injection of an adeno-associated virus vector into nuclei with divergent connections results in widespread vector distribution in the brain and global correction of a neurogenetic disease. J Neurosci, 2007, 27: 9928–9940.
11. Meijer DH, Maguire CA, LeRoy SG, *et al*. Controlling brain tumor growth by intraventricular administration of an AAV vector encoding IFN-beta. Cancer Gene Ther, 2009, 16: 664–671.
12. Samaranch L, Salegio EA, San Sebastian W, *et al*. Strong cortical and spinal cord transduction after AAV7 and AAV9 delivery into the cerebrospinal fluid of nonhuman primates. Hum Gene Ther, 2013, 24: 526–532.
13. Chang M, Copper JD, Sleat DE, *et al*. Intraventricular enzyme replacement improves disease phenotypes in a mouse model of late infantile neuronal ceroid lipofuscinosis. Mol Ther, 2008, 16: 649–656.
14. Zhuang X, Xiang X, Gizzle W, *et al*. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. Mol Ther, 2011, 19: 1769–1779.
15. Hocquemiller M, Giersch L, Audrain M, *et al*. Adeno-associated virus-based gene therapy for CNS diseases. Hum Gene Ther, 2016, 27: 478–496.
16. Zhang H, Yang B, Mu X, *et al*. Several rAAV vectors efficiently cross the blood-brain barrier and transduce neurons and astrocytes in the neonatal mouse central nervous system. Mol Ther, 2011, 19: 1440–1448.
17. Pulicherla N, Shen S, Yadav S, *et al*. Engineering liver-detargeted AAV9 vectors for cardiac and musculoskeletal gene transfer. Mol Ther, 2011, 19: 1070–1078.
18. Deverman BE, Pravdo PL, Simpson BP, *et al*. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. Nat Biotechnol, 2016, 34: 204–209.
19. Bartus RT, Baumann TL, Siffert J, *et al*. Safety/feasibility of targeting the substantia nigra with AAV2-neurturin in Parkinson patients. Neurology, 2013, 80: 1698–1701.
20. Mittermeyer G, Christine CW, Rosenbluth KH, *et al*. Long-term evaluation of a phase 1 study of AADC gene therapy for Parkinson's disease. Hum Gene Ther, 2012, 23: 377–381.
21. Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol, 2009, 187: 761–772.
22. Kügler S, Kilic E, Bähr M. Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. Gene Ther, 2003, 10: 337–347.
23. Foust KD, Nurre E, Montgomery CL, *et al*. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. Nat Biotechnol, 2009, 27: 59–65.
24. Ojala DS, Amara DP, Schaffer DV. Adeno-associated virus vectors and neurological gene therapy. Neuroscientist, 2015, 21: 84–98.
25. Chan KY, Jang MJ, Yoo BB, *et al*. Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. Nat Neurosci, 2017, 20: 1172–1179.
26. Gray SJ, Matagne V, Bachaboina L, *et al*. Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. Mol Ther, 2011, 19: 1058–1069.
27. McLean JR, Smith GA, Rocha EM, *et al*. Widespread neuron-specific transgene expression in brain and spinal cord following synapsin promoter-driven AAV9 neonatal intracerebroventricular injection. Neurosci Lett, 2014, 576: 73–78.
28. Li DP, Zhou JJ, Zhang J, *et al*. CaMKII regulates synaptic NMDA receptor activity of hypothalamic presympathetic neurons and sympathetic outflow in hypertension. J Neurosci, 2017, 37: 10690–10699.
29. Chira S, Jackson CS, Oprea I, *et al*. Progresses towards safe and efficient gene therapy vectors. Oncotarget, 2015, 6: 30675–30703.

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