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To explore the therapeutic effects of shRNA1 and shRNA4 on Parkinson's disease mice based on the mutation of the SNCA gene of α-synuclein

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Abstract:

Based on the SNCA gene mutation of α-synuclein, the therapeutic effects of shRNA1 and shRNA4 on Parkinson's mice were explored, and the migration mechanism of α-synuclein was explored. Methods: $SNCA^{+/+}$ mice were used to induce the production of α-synuclein using tuberculin pff to establish Parkinson's mouse models. AAV-shRNA1 and AAV-shRNA4 were used to set up control and experimental groups in different brain regions and at different times. The open field test, tail suspension test and immunofluorescence were used to explore the therapeutic effect of shRNA4 and the migration mechanism of α-synuclein. Results: ShRNA4 has a significant inhibitory effect on P-α-syn in the brains of Parkinson's mice compared to shRNA1, and it also shows significant behavioral improvement. The α-synuclein in the olfactory bulb (OB) has undergone whole-brain invasion, while the α-synuclein in the dorsal striatum (CPu) has only undergone invasion in downstream brain regions. Conclusion: Tail vein injection of shRNA4 can have a significant inhibitory effect on α-synuclein and symptomatic treatment in Parkinson's mice, and the earlier the treatment is initiated, the better the effect.

Keywords: shRNA;α-synuclein; Parkinson's disease; SNCA gene.

1.Introduction

Parkinson's disease is a common neurological disease in middle-aged and elderly people. The main pathological changes of PD are the degeneration and death of dopaminergic neurons in the substantia nigra of the midbrain, a significant decrease in dopamine levels in the striatum, and the appearance of eosinophilic inclusions in the cytoplasm of residual neurons in the substantia nigra, namely Lewy Body. Lewy bodies are mainly produced by the aggregation of α-synuclein. 90% of the components of LB areα-syn, while α-syn only accounts for 4% in normal human brain tissue. PD has a long course, many complications, and is difficult to cure. Studies have shown that the incidence of PD is on the rise. There are more than 2.5 million PD patients in China, and about 100,000 new cases reported each year. Since the 1960s, despite great breakthroughs in the study of the pathogenesis of PD, there are not many drugs for the treatment of PD. Currently, levodopa replacement therapy is mainly used to treat PD, but it cannot prevent the progression of PD. The therapeutic effect begins to decline after 3 to 5 years. Not only are there early adverse reactions (anorexia, nausea, dizziness, mental disorders and dyskinesia, etc.), but long-term use can also lead to "switching phenomenon", end-of-dose phenomenon and muscle tension movement disorders. Studies have shown that the incidence of motor complications is 40% and 70% after 5 years and 15 years of using compound levodopa, respectively. Therefore, it is urgent to seek new treatment methods.

Studies have shown that SNCA is the first gene reported to be associated with the onset of familial PD. The onset of PD after its mutation is often characterized by chromosomal dominant inheritance, and it can also increase the susceptibility of sporadic PD. Therefore, this experiment will use transgenic TG-SNCA+/- mice as model animals. SNCA mainly encodes α-syn, which is located on chromosome 4q21-22. It can cause the early onset of PD and affect the progression of PD through single point mutations (mutation sites such as A53T, A30P, E64L, etc.), double and triple replication, frameshift mutations, etc., so gene therapy is a more ideal and thorough treatment method. The exploration of gene therapy has never been interrupted for decades, but it often requires the reprinted plasmid to be injected into the brain, which will cause mechanical damage to the brain, so the research progress of gene therapy was limited.

Recombinant AAVs (rAAVs) are commonly used vectors

for in vivo gene transfer and are also a promising therapeutic vector. However, AAVs that can deliver genes to specific cell populations efficiently and non-invasively are needed. Current gene delivery methods (such as intraparenchymal surgical injection) are invasive, and alternative methods such as intravenous injection require high viral doses and provide relatively inefficient target cell transduction. It was not until 2019 that Viviana Gradinaru et al. developed the Cre-based AAV Targeted Evolution (CREATE) technology for designing and screening AAV capsids that can more efficiently transfer genes to specific cell types through blood vessels. Tail vein injection of specific adeno-associated viruses (AAVs) can break through the blood-brain barrier and infect brain tissue, which is also one of the basic theories and means of our experiment. shRNA (short hairpin RNA), which can produce siRNA endogenously for a long time after transfection and stably interfere with the target gene, so AAV-U6-α-syn-shRNA-hsyn-tdT will be used as a recombinant plasmid for the treatment of Parkinson's in this experiment.

In order to find a gene therapy method that is more suitable for clinical use, this experiment will use TG-SNCA mice to create a mouse Parkinson's model using purified α-syn-pff and give packaged and transfected AAV-shRNA for treatment. Through immunofluorescence staining, open field test, and tail suspension test, the effects of shRNA1 and shRNA4 on mouse p-α-syn at the animal molecular level and overall level will be explored, hoping to provide certain data for the treatment and research of Parkinson's.

2.Method and material

Model Building

(1)Selection of experimental mice: WT mice and TG-SNCA$^{+/-}$ mice were selected from the Kunming Institute of Zoology. All animal experiments were approved by the Animal Ethics Committee. All mice were about 8 weeks old and weighed 18-24g. All experimental mice were placed in the experimental environment for at least 2 weeks to adapt to the experimental environment. The mice had free access to water and food.

(2)Model establishment method: The animals were anesthetized by intraperitoneal injection of pentobarbital (35mg/Kg), and then the mouse venous blood was collected by tail-cutting method and added to the EP tube soaked with heparin. The rectal temperature was maintained at 37.5℃

using a feedback-controlled heating pad. The mice were placed in a stereotaxic frame and a hole (about 1 mm in diameter) was drilled in the skull near the right coronal suture. A 26-gauge needle was inserted stereotaxically into the right olfactory bulb (coordinates: X=-0.75 mm, Y=+5.10 mm, Z=-1.00 mm) or the right striatum (coordinates: X=-1.70 mm, Y=-0.86 mm, Z=-2.40 mm) and PFF was infused into the experimental group mice at a rate of 1 μL/min using a micro infusion pump. The needle was removed, the drill hole was filled with bone wax, and the skin incision was sutured. Two months later, the tail vein was injected with post-AAV.

(3)AAV-shRNA virus production:

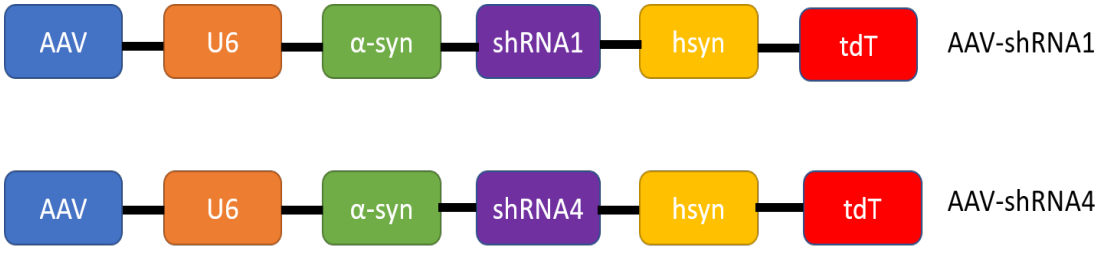

Fig 1.AAV Viral structure

Immunofluorescence analysis of the effect of AAV-shRNA on p-α-syn and its migration mechanism in the brain

(1)Perfusion and fixation of mouse brain

Place 0.9% saline in a conical flask and place it in a 37℃

a) water bath.

b) Prepare the mice and give them an intraperitoneal injection of 20% urethane (0.1 ml/10 g).

c) Turn on the perfusion pump and quickly flush the tube wall with 0.9% saline.

d) Press the air valve to exhaust the gas, and perfuse the pump at 4.5rpm

e) Adjust the perfusion pump to 1.0rpm, place it in 0.9% saline, and fix the limbs of the anesthetized mouse on the foam board

f) Cut open the mouse's abdominal cavity, push the intestines apart, cut open the diaphragm, expose the chest cavity, turn the ribs up, fix them on the foam board, and expose the heart

g) Cut off a piece of liver, insert the infusion needle into the apex of the mouse's left ventricle, control the depth and strength of the needle, and fix the infusion device with pointed forceps

h) Infusion pump Place in 0.9% saline, 5.0rpm, 10min

i) Place the infusion pump in 4% PFA, 4.5rpm, 50min

j) Observe whether the mouse perfusion is successful. If successful, prepare to remove the brain

k) Cut the mouse skull from the upper edge of the forelimbs
l) Cut the scalp, peel it open on both sides, and remove excess muscle
m) Use a lancet to gently scratch along the middle suture of the mouse skull, and the front end can be cut off
n) Use sharp forceps to peel the skull
o) If post-fixation is not necessary, the brain tissue can be placed in 30% sucrose for 24h (until it sinks to the bottom)
(2)Frozen sections
a) Turn on the slicer, adjust the temperature of the chamber to -20℃, the head to -21℃, the slice thickness to 40μm, and put the blade into the knife groove
b) Flatten the olfactory bulb of the mouse head, put it into the sample holder, ensure symmetry, embed it with an embedding agent, and put it into the slicer for 20 minutes
c) Clamp the sample holder on the head, adjust the front and back distance, and prepare for slicing
d) Discard the spinal cord, start slicing from the medulla oblongata, and try to slice at a uniform speed
e) Put 5 brain slices in one hole, and check the approximate position with the brain map. Put the brain slices into a 24-well plate filled with PBS
f) After cutting, remove the sample holder, clean the table, and put the 24-well plate into a 4℃ refrigerator
g) After cutting, adjust the chamber temperature to -10℃ and wait. If you don't need the instrument, you can click "OFF" and "Head -" to turn off the machine and lock the machine
(3)Immunofluorescence steps:
a) Block overnight with 10% goat serum
b) Incubate with primary antibody (Rabbit-MPO, Solarbio, 1:300) at room temperature for 6 hours, protect from light
c) Wash three times with 0.01M pH7.2 PBS, 8 minutes each time
d) Incubate with secondary antibody (Goat-Rabbit, Solarbio, 1:250) at room temperature for 2 hours, protect from light
e) Wash three times with 0.01M pH7.2 PBS, 8 minutes each time
f) DAPI staining, incubate at room temperature for 10 minutes, protect from light
g) Wash once with 0.01M pH7.2 PBS, 8 minutes each time
h) Seal with PBS-anti-fluorescence quencher, protect from light, and place in a refrigerator at 4℃
Using behavioral experiments to verify the therapeutic effect of shRNA on Parkinson's mice
(1)Open field test
The experimental device is a 50cm×50cm×50cm cubic box made of opaque plastic, with a white bottom and inner wall. The mouse is placed in a specific corner facing the central area and the timer starts. The mouse's activities within 15 minutes are automatically recorded. The open field in the video is divided into a central area and a peripheral area through specific behavioral software, with the central area accounting for 25%, and the mouse movement is tracked and identified simultaneously. The movement distance, movement speed, static time, and the time the mouse spends in the central area are observed to evaluate the mouse's exercise ability.
(2)Tail suspension test
The mouse was suspended by its tail, and the movement of its hind limbs was recorded within 15 seconds. The clasping condition and muscle strength of the hind limbs were observed.
Statistical analysis
All data were expressed as Mean ± SEM, unpaired student t-test was used for comparison between two groups, one-way ANOVA after post hoc test was used for comparison between multiple groups, and $p < 0.05$ was considered statistically significant. The statistical graph drawing software was Prism8, and the flow cytometry analysis software was Flow-Jo.

3. Results

Results of shRNA treatment 1.5 months after olfactory bulb injection of PFF
shRNA1 is the control group, and the corresponding shRNA4 is the test group. From the behavioral results, we can see that the test group has obvious behavioral therapeutic effects. In the open field test, the movement distance and time of mice were significantly increased, and in the tail suspension test, the hind limb muscle strength of mice was also significantly improved.

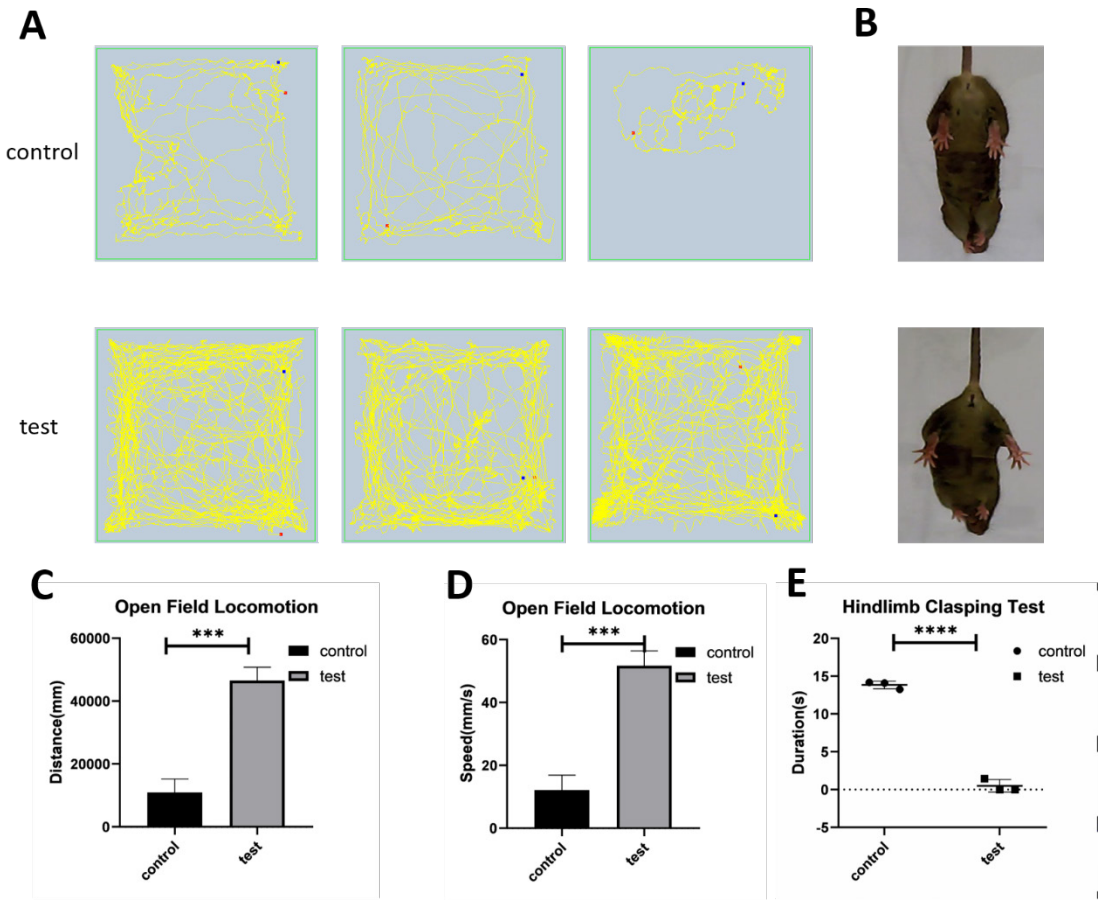

Fig2.Olfactory bulb injection of pff and shRNA treatment 1.5 months later.

A. Open field test results, the marked follow-up point is the mouse's center of gravity, Each recording time is 15 minutes. B. Result graph of tail suspension test, each recording time is 15 seconds. C. Statistical graph of mouse distance in open field test, *** is $P < 0.001$. D. Statistical graph of mouse speed in open field test, *** is $P < 0.001$. E. The time of mouse hind limb clasping in the tail suspension test, ** **is $P < 0.0001$.

Results of shRNA treatment 2.5 months after olfactory bulb injection of PFF

shRNA1 is the control group, and the corresponding shRNA4 is the test group. From the behavioral results, we can see that the test group has obvious behavioral therapeutic effects. In the open field test, the movement distance and time of mice were significantly increased, and in the tail suspension test, the hind limb muscle strength of mice was also significantly improved.

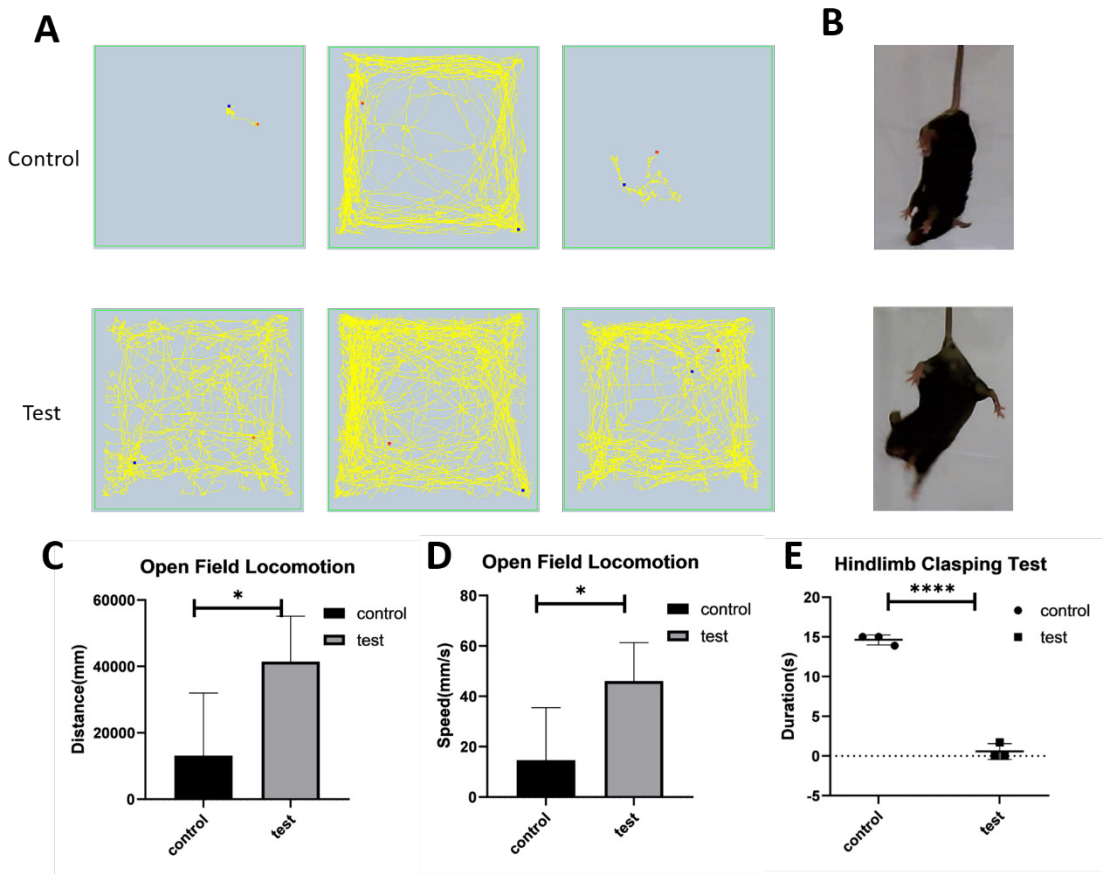

Fig3.Olfactory bulb injection of PFF followed by shRNA treatment 2.5 months later.

A. Result of the open field test, the marked follow-up point is the mouse's center of gravity, and each recording time is 15 minutes. B. Result of tail suspension test, each recording time is 15 seconds. C. Statistical graph of mouse distance in open field test, * is $P < 0.05$. D. Statistical graph of mouse speed in open field test, * is $P < 0.05$. E. The time of mouse hind limb clasping in the tail suspension test, * * * * is $P < 0.0001$.

Results of shRNA treatment 1.5 months after striatal injection of PFF

shRNA1 is the control group, and the corresponding shRNA4 is the test group. We can see from the behavioral results that the test group has obvious behavioral therapeutic effects. The movement distance and time of mice in the open field test have increased significantly, and the hind limb muscle strength of mice in the tail suspension test has also improved significantly. It is particularly important to note that in the third group of animals, the test group and the control group showed opposite results, so we eliminated them after the experiment.

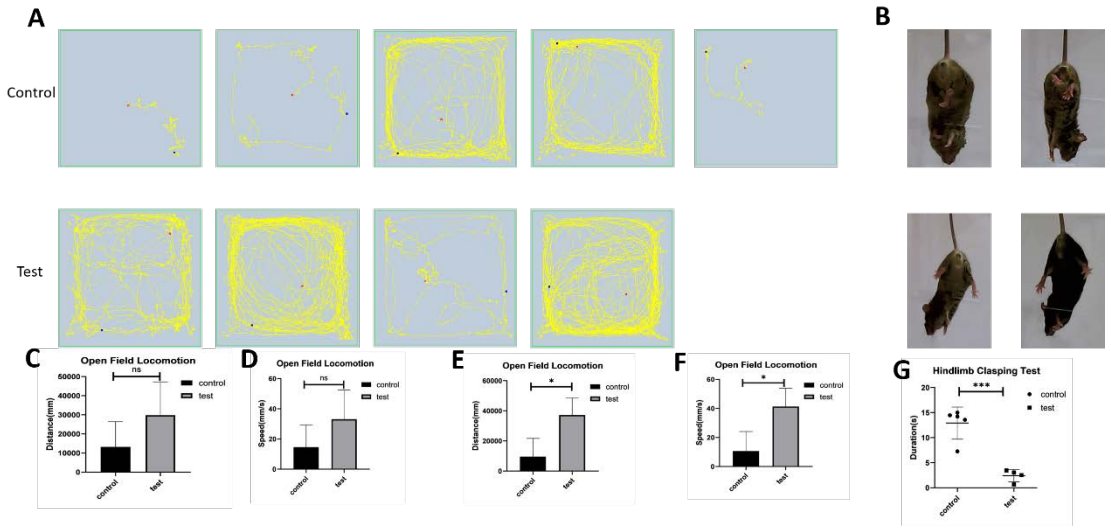

Fig4.Striatal injection of PFF 1.5 months after shRNA treatment.

A. Result graph of the open field test, the marked follow-up point is the mouse's center of gravity, and each recording time is 15 minutes. B. Result graph of tail suspension test, each recording time is 15 seconds. C. Statistical graph of mouse distance in open field test, this graph is the group with abnormal results not removed (P3), ns is P>0.05. D. Statistical graph of mouse speed in open field test, this graph is the group with abnormal results not removed (P3), ns is P>0.05. E. Statistical graph of mouse distance in open field test, this graph is the group with abnormal results removed (P3), * is P<0.05. F. Statistical graph of mouse speed in open field test, this graph is the group with abnormal results removed (P3), * is P<0.05. G. Mouse hind limb clasping time in tail suspension test, * * * is P<0.001.

P-α-syn aggregation in the mouse brain after injection of pff at SNC

The results show that P-α-syn was successfully induced after injection of pff into the substantia nigra and striatum (SNC).

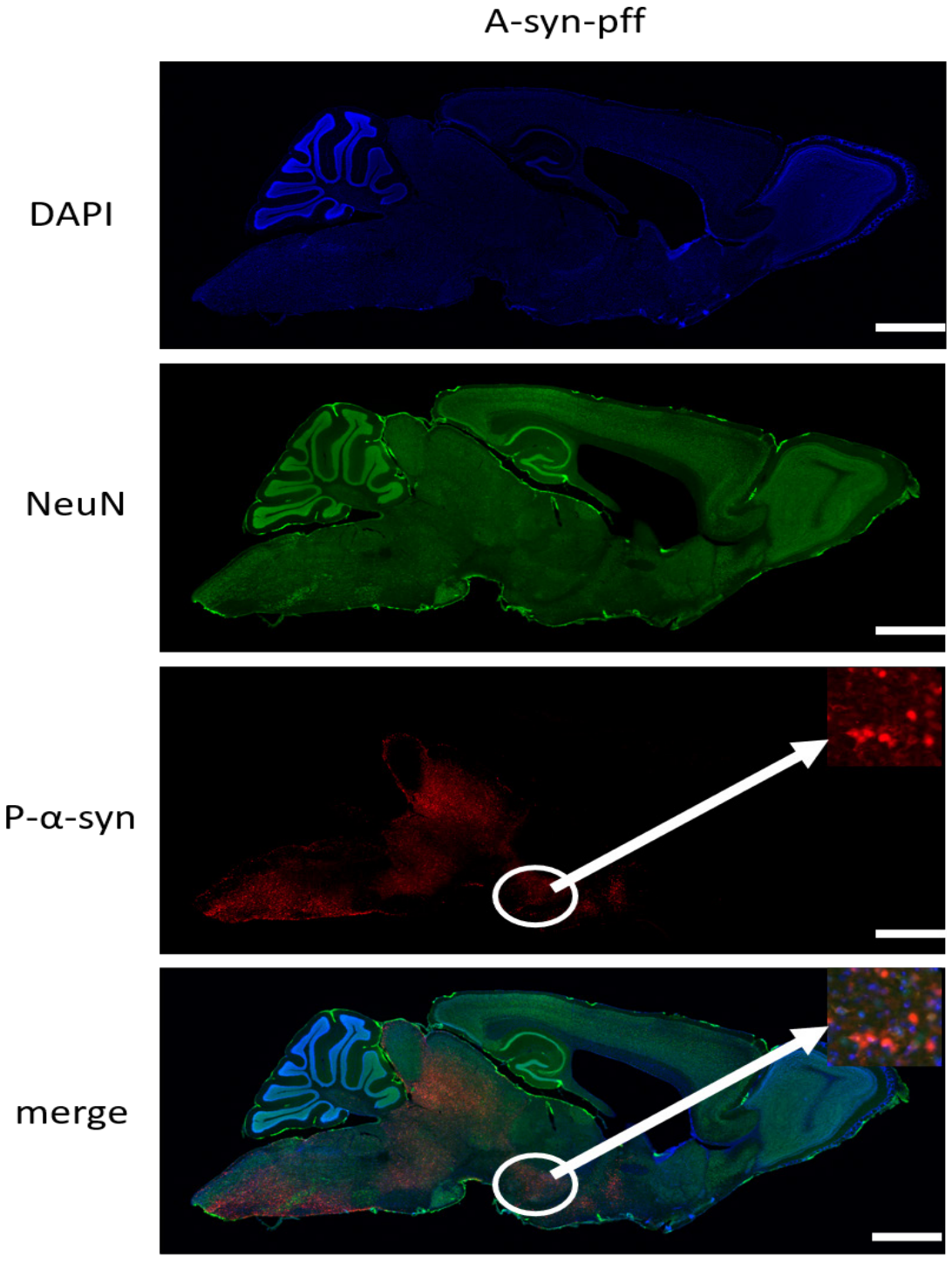

Fig5.Aggregation of P-α-syn in the mouse brain after injection of pff at SNC.

The blue color represents the cell nucleus labeled by DAPI, the green color represents the neuronal-glial cells in the brain labeled by Goat-Mouse-NeuN-488, and the red color represents the P-α-syn in the brain labeled by Goat-Rabbit-p-α-syn-546.

Brain and liver infection after tail vein injection of shRNA

As the largest metabolic organ, after AAV-shRNA was injected into the tail vein, no AAV was detected in the liver, but AAV was present in large quantities in the brain, indicating that this AAV can migrate to the brain in a targeted manner.

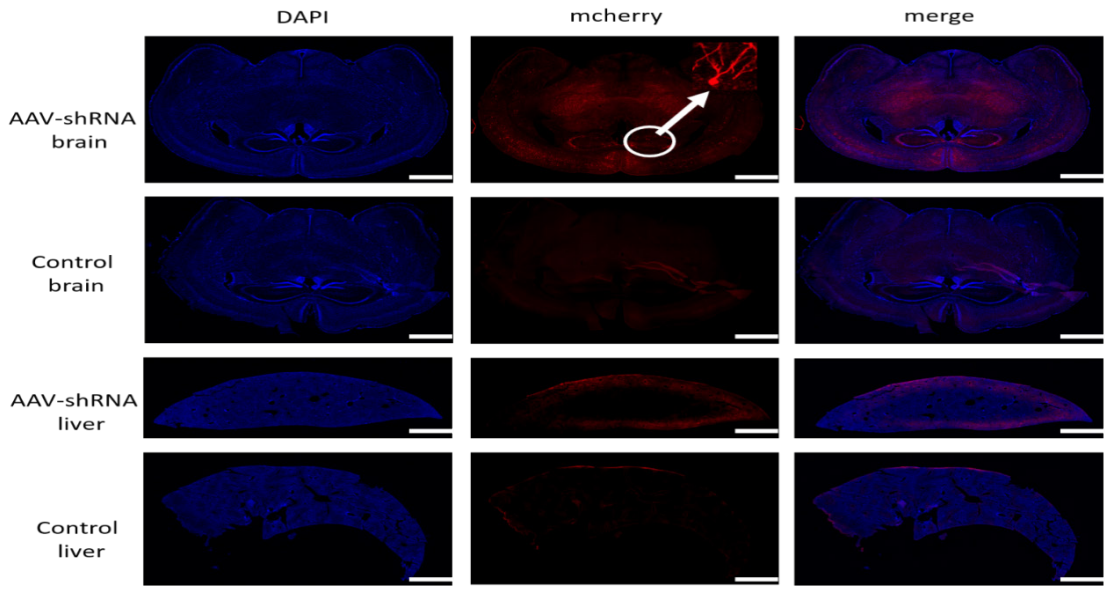

Fig6. Brain and liver infection after tail vein injection of shRNA.

The blue color represents the cell nucleus labeled with DAPI, and the red color represents the enhanced signal of tdT labeled with Goat-Rabbit-mcherry-546.

Treatment of shRNA by tail vein injection after pff injection in CPu

In addition to comparing the shRNA1 and shRNA4 groups, we also tested the infection of the left and right brains (the injection site was on the right side). The results showed that P-α-syn was significantly inhibited in the shRNA4 group, but the changes between the left and right brains were not significant.

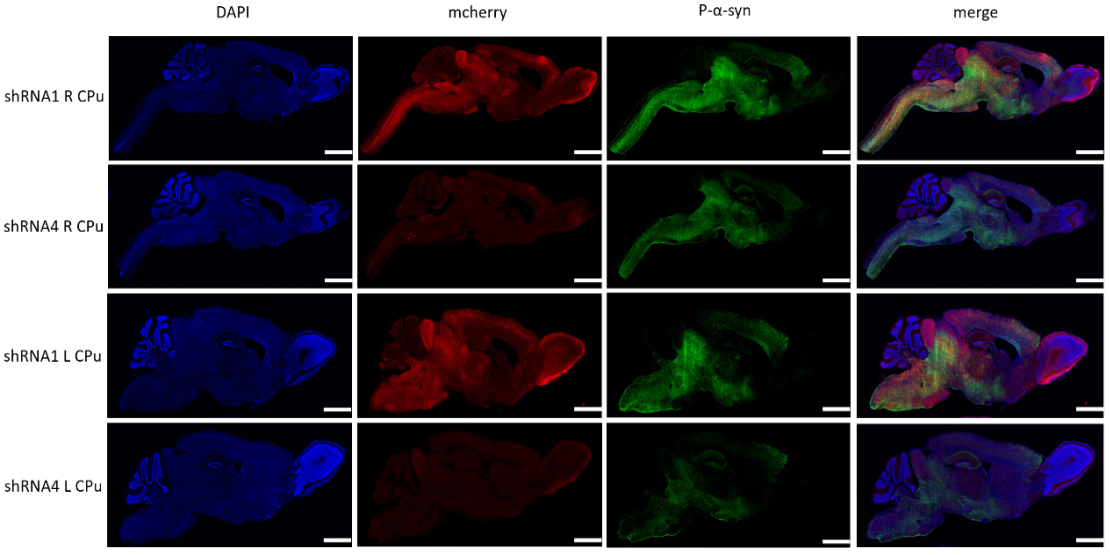

Fig7. Treatment of shRNA injected into the tail vein after CPu injection of pff.

The pff injection position of the control group was the right CPu, and AAV-U6-α-syn-shRNA1-hsyn-tdT was injected into the tail vein. The pff injection position of the test group was the left CPU, and AAV-U6-α-syn-shRNA4-hsyn-tdT was injected into the tail vein. Blue is DAPI labeled nuclei, red is Goat-Chicken-mcherry-546 labeled tdT enhanced signal, and green is Goat-Rabbit-p-α-syn-488 labeled P-α-syn in the brain.

Treatment of shRNA by tail vein injection after pff injection in OB and CPu

In addition to comparing shRNA1 and shRNA4 in OB and CPu, we also tested the infection of the left and right brains (the injection site was on the right side). The results showed that P-α-syn was significantly inhibited in the shRNA4 group, but the changes in the left and right brains were not significant. However, the difference was

that the mice in the OB injection group clearly had P-α-syn throughout the brain, but the mice in the CPu injection group only had P-α-syn in the downstream brain areas of CPu.

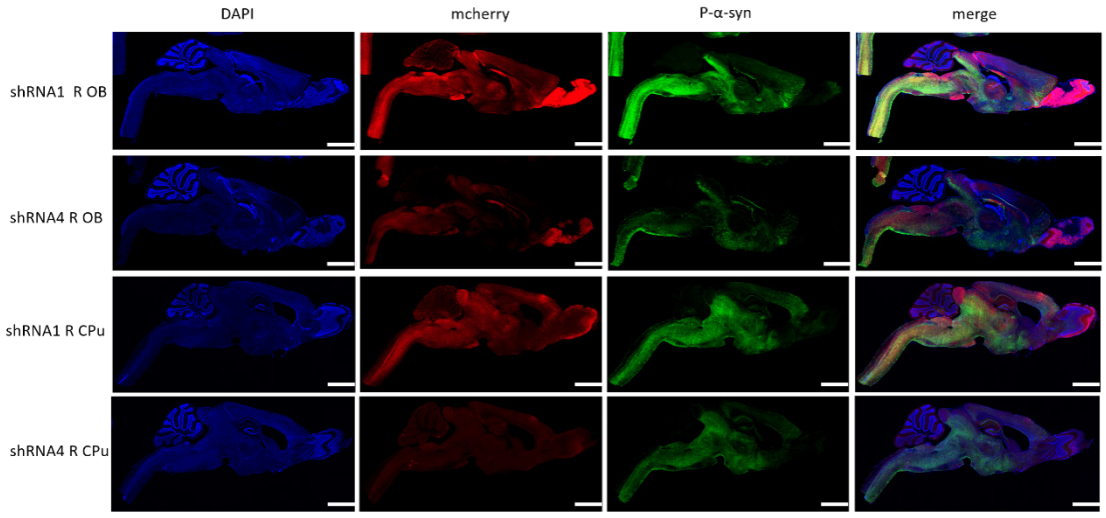

Fig8. Treatment of shRNA by tail vein injection after pff injection into OB and CPU.

The pff injection position of the control group was the right CPu, and AAV-U6-α-syn-shRNA1-hsyn-tdT was injected into the tail vein. The pff injection position of the test group was the left CPu, and AAV-U6-α-syn-shRNA4-hsyn-tdT was injected into the tail vein. Blue is DAPI labeled nuclei, red is Goat-Chicken-mcherry-546 labeled tdT enhanced signal, and green is Goat-Rabbit-p-α-syn-488 labeled P-α-syn in the brain.

4. Discussion

Combining relevant literature with our results, it is not difficult to find that tuberculin (pff) can successfully induce the production of P-α-syn in the brain of SNCA+/+ mice, which is a basic condition for the success of our experiment. Another basic condition is that AAV-shRNA can be detected in the brain after peripheral vein (tail vein) injection. What is particularly gratifying is that we did not detect the presence of AAV-shRNA in the liver, the largest metabolic organ in the body, which also shows that the AAV-shRNA we use can be directed to the brain to play a role.

After meeting our basic conditions, we set up experimental and control groups for OB injection for 1.5 months, experimental and control groups for OB injection for 2.5 months, and experimental and control groups for CPu injection for 1.5 months. We conducted two neurobehavioral experimental analyses on these six groups: an open-field test and a tail suspension test. The results show that the test group of shRNA4 showed a more obvious therapeutic effect compared with the control group of shRNA1, and it can be seen that in the same OB group, the 1.5-month treatment effect was significantly better than that of 2.5 months, and the 1.5-month group of OB and CPu was significantly better than that of CPu group. This also proves that the effect is best in early treatment and in the more anterior brain areas (because the most common early symptom of Parkinson's patients is hyposmia). Among them, the third column of data in our CPu group needs to be discussed in particular. After our discussion, we believe that the titer of AAV injected into this group of mice may be impure, so the opposite result appeared, but only appeared in this group, so it can be eliminated.

To further explore the specific relationship, we used sagittal brain slices for immunofluorescence triple staining, and the results were consistent with the behavioral experiments. The shRNA4 group had a significant therapeutic effect, both in the OB group and the CPu group. Among the gratifying results, we found that after the injection of pff into the right brain region to induce P-α-syn, the same P-α-syn appeared in the contralateral brain region, and after the injection of pff into the OB to induce P-α-syn, P-α-syn appeared in the whole brain, but after the injection of pff into the CPU to induce P-α-syn, P-α-syn only appeared in its downstream brain region, which shows that the migration of α-syn is in a front-to-back order, which also corresponds to the process of Parkinson's patients from early olfactory impairment and other minor changes to later tremor atrophy.

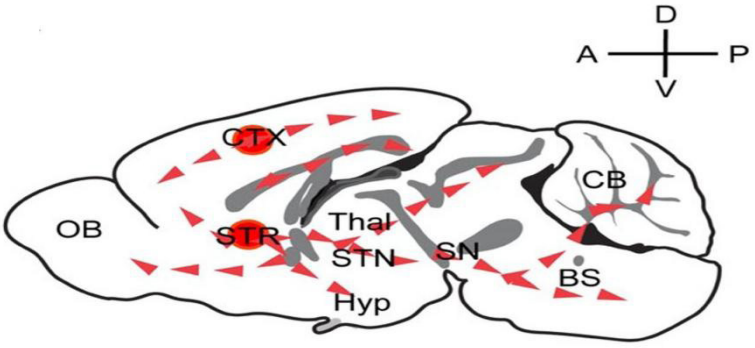

Fig9.Possible invasion mechanism of α-syn in the brain

References

[1]. Kum WF, Durairajan SS, Bian ZX, et al. Treatment of idiopathic Parkinson's disease with traditional Chinese herbal medicine: A randomized placebo-controlled pilot clinical study[J], Evid Based Complement Alternat Med,2011,2011:724353.

[2]. Chan, K. Y. et al. Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems. Nat. Neurosci. 20, 1172–1179 (2017).

[3]. Deverman, B. E. et al. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. Nat. Biotechnol. 34, 204–209 (2016).

[4]. Bedbrook, C. N., Deverman, B. E. & Gradinaru, V. Viral strategies for targeting the central and peripheral nervous systems. Annu. Rev. Neurosci. 41, 323–348 (2018).

[5]. Baba, M., S. Nakajo, P.H. Tu, T. Tomita, K. Nakaya, V.M.Y. Lee, J.Q. Trojanowski, and T. Iwatsubo. 1998. Aggregation of alphasynuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am. J. Pathol. 152:879–884.

[6]. Kelvin C. Luk, Victoria M. Kehm, Bin Zhang, Patrick O'Brien, John Q. Trojanowski, and Virginia M.Y. Lee, Intracerebral inoculation of pathological α-synuclein initiates a rapidly progressive neurodegenerative α-synucleinopathy in mice, J.E.M.209(5):975-986(2013).

[7]. Yardeni, T., Eckhaus, M., Morris, H. D., Huizing, M. & Hoogstraten-Miller, S. Retro-orbital injections in mice. Lab Anim. 40, 155–160 (2011).

[8]. Callaway EM, Luo L. 2015.Monosynaptic circuit Tracing with glycoprotein-deleted rabies viruses. J.Neurosci. 35:8979–85