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Direct intrathecal injection of recombinant adeno-associated viruses in adult mice --Manuscript Draft--

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TITLE:

Direct Intrathecal Injection of Recombinant Adeno-Associated Viruses in Adult Mice

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KEYWORDS:

Intrathecal, AAV, gene transduction, central nervous system, lidocaine hydrochloride, EGFP

SUMMARY:

Here we present a direct intrathecal injection technique using 1% lidocaine hydrochloride in a viral solution to ensure efficient adeno-associated virus delivery to small animals and establishment of a scoring system to predict transduction efficiency in the central nervous system according to the degree of transient weakness induced by lidocaine.

ABSTRACT:

Intrathecal (IT) injection of adeno-associated virus (AAV) has drawn considerable interest in CNS gene therapy by virtue of its safety, noninvasiveness, and excellent transduction efficacy in the CNS. Previous studies have demonstrated the therapeutic potency of AAV-delivered gene therapy in neurodegenerative disorders by IT administration. However, high rates of unpredictable failure due to the technical limitation of IT administration in small animals have been reported. Here, we established a scoring system to indicate the success extent of lumbar puncture in small animals by adding 1% lidocaine hydrochloride into the injection solution. We further show that the extent of transient weakness following injection can predict the transduction efficiency of AAV. Thus, this IT injection method can be used to optimize

therapeutic trials in mouse models of CNS diseases that afflict wide regions of the CNS.

INTRODUCTION:

AAV can mediate long-term and widespread gene expression in the CNS transduction with few side effects, and therefore has become one of the most promising vehicles for gene therapy to treat CNS diseases including amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), Alzheimer's disease (AD), lysosomal storage diseases (LSD), Gaucher disease (GD), and neuronal ceroid lipofuscinosis (NCL)¹. Presently, more than 100 AAV serotypes have been isolated from humans and animals. Among these, at least 12 have been used in preclinical and clinical trials, including the most commonly used gene vectors such as AAV1, 2, 4, 5, 6, 8, 9, rAAVrh.8, and rAAVrh.10¹⁻⁶.

Different CNS diseases require different AAV delivery strategies due to the various affected CNS regions and cell types. The CNS regions and cell types that AAV can transduce varies depending on the serotype as well as delivery method. For example, rAAVrh10 has been shown to transduce predominantly astrocytes when delivered by systemic intravenous injection (IV), whereas it transduced both neurons and glia when delivered by intrathecal injection^{4,7}. Additionally, parenchyma injection resulted in local transduction to the vicinity of the injection site, whereas injection into the cerebrospinal fluid (CSF) through intraventricular or intrathecal injection resulted in widespread CNS transduction⁸. Studies have also demonstrated therapeutic potency of AAV-delivered gene therapy in neurodegenerative disorders by IT administration⁹⁻¹¹. In diseases that affect broad areas of the CNS such as ALS, intrathecal injection into the CSF has been shown to cover most areas that are afflicted by the disease with a lower dose, compared to a systemic delivery method^{4,10}. Recent studies have also shown that lumbar puncture can be used to inject AAV in mouse models for ALS, which avoids potential injuries associated with laminectomy and intrathecal catheterization⁴.

Experimental direct lumbar puncture was first used to deliver agents, especially anesthetics, to the spinal cord for analgesia and anesthesia in 1885^{12,13}. In this report, we illustrate the lumbar puncture IT injection method in adult mice with the aid of 1% lidocaine hydrochloride, a local amide-derived anesthetic, in the injection solution to evaluate and monitor injection quality. Successful injections were marked by lidocaine-induced transient paralysis, whereas failed injections did not show this behavior. We classified the level of transient weakness as one of five grades to help predict the injection efficiency. Finally, we show that the rAAVrh10 transduction level may be predicted by the grade of paralysis. Therefore, this intrathecal AAV delivery method can be used to enhance AAV-mediated gene-delivery for experimental therapy of CNS diseases.

PROTOCOL:

FVB/NJ mice were bred in the animal facility of Key Laboratory of Hebei Neurology. All mouse experiments were approved by the Second Hospital of Hebei Medical University Ethics Committee and carried out according to the regulations of laboratory animal management promulgated by the Ministry of Science and Technology of the People's Republic of China.

1. Preparation of 20% Lidocaine Hydrochloride Stock Solution

1.1 Weigh 2 g of lidocaine hydrochloride. Add 5-6 mL of sterile water and vortex gently. Increase the volume to a total of 10 mL with sterile water.

1.2 Filter the stock solution through 0.2-micron filters. Aliquot the stock solution, 1 mL per 1.5 mL microcentrifuge tube, and seal it with a sealing film. Store at 4 °C.

2. Direct Intrathecal AAV Delivery in Awake Mice

2.1 Wipe the work area using sterile gauze with 70% ethanol and prepare the required supplies as mentioned in **Table 1**.

2.2. Prepare 100 µL of AAVrh.10/1% lidocaine hydrochloride complex by adding 95 µL of rAAVrh10 stock solution (5×10^{12} genome copies/mL) into a sterile 200 µL microcentrifuge tube, and add 5 µL of 20% lidocaine hydrochloride stock solution. Mix well by pipetting up and down. Then, store the virus solution on ice (4 °C).

Note: 8 µL per mouse⁴ will be used.

2.3 Preparing the syringe with AAV solution for injection

2.3.1 Assemble a 25 µL Hamilton syringe with a 27-gauge needle and align the beveled tip of the needle with the volumetric scale on the syringe.

2.3.2 Draw 8 µL with 4×10^{10} genome copies of the virus solution into the syringe gently. Make sure to remove air bubbles.

2.4 Preparing the mouse

Note: Male or female FVB/NJ mice (30-70 days old) were used in this study. IT injection was operated in the hood.

2.4.1 Sterilize the work area in a hood with 70% ethanol. Put the awake mouse (male or female, 30-70 days old, 13-20 g weight) on a bedpiece in a prone position in the hood. Cover the upper body with sterile gauze to calm the mouse and avoid being bitten.

2.4.2 Fix the animal by gripping appropriately and firmly on its pelvic girdle with a thumb on one side and forefinger/middle finger on the other side. Keep the skin between bilateral pelvic girdles taut with the thumb and forefinger. Hold gently on the upper body of the animal with the palm.

2.4.3 Shave the fur on its back between the bilateral pelvic girdles, then sterilize the skin surface with an iodide-based scrub and 70% ethanol.

2.5 Intrathecal Injection¹²

2.5.1 Feel the intervertebral space along the middle line between the bilateral pelvic girdles with a thumb or forefinger of the other hand and press an indentation with a fingernail to indicate the L5-L6 intervertebral space (locate the injection site).

2.5.2 Rotate the base of the tail slightly and gently to indicate the midline of the spine. Adjust the needle bevel towards the head of the animal before injection (mentioned in step 2.3.1).

2.5.3 Make sure that the animals are fixed firmly and align the needle along the midline of spine.

2.5.4 Insert the needle gently and vertically (or tilt slightly 70-80°) in the intersection of indentation and keep the syringe in a central sagittal plane. Reduce the angle to approximately 30° slowly when it connects the bone, then slip the needle into the intervertebral space.

Notes: An evident sudden tail flick is a sign of successful entry into the intradural space. Once the needle enters the intervertebral space, the needle tip will feel firmly clamped. The 27G needle used in this study is suited for IT delivery in mice but not rats.

2.5.5 Inject the vector solution (mentioned in step 2.2). Start the timer and inject 8 μ L of the vector solution at a speed of 1 μ L/4 s. Retain the needle approximately 1 min after finishing delivery. Withdraw the needle with gentle rotation to avoid leaking.

2.5.6 Score the transient weakness of the mouse limbs immediately after delivery to evaluate the injection quality⁴.

Notes: The standard is as follows⁴. Score 0: no weakness; score 1: minor weakness of the hind limbs without gait abnormality; score 2: moderate weakness of the hind limbs with obvious gait abnormality; score 3: complete paralysis of the hind limbs; score 4: complete paralysis of the hind limbs, shortness of breath, and moderate weakness of the fore limbs; and score 5: complete paralysis of all four limbs and evident shortness of breath.

2.5.7 Move the mouse back to the cage for recovery from paralysis.

2.6 Clean-up

2.6.1. Flush the syringe with 1 mL sterile water. Sort laboratory supplies and collect all non-disposable materials for autoclaved sterilization. Clean the bench with 70% ethanol.

3. Tissue Preparation for Immunohistochemical Staining

3.1 Tissue collection

3.1.1 Anesthetize mice at 21 days post-injection with 3% chloral hydrate (0.1 mL/10 g) deeply by intraperitoneal injection.

3.1.2 Perfuse transcardially with 20 mL of ice-cold 0.01 M PBS (NaCl 147 mM; NaH₂PO₄ 1.9 mM; K₂HPO₄ 8.1 mM, pH 7.4) firstly, then 4% ice-cold paraformaldehyde (in 0.01 M PBS) with pump (10 mL/min for 1 min, then 5 mL/min for 9 min) .

CAUTION: Paraformaldehyde is carcinogenic and toxic. Handle it only in the fume hood while wearing gloves.

3.2 Dissection of the spinal cord and brain

3.2.1 Fix the limbs and head of each animal in a prone position on a foam box cover with syringe needles, then strip and remove the skin from the head to sacrum with scissors.

3.2.2 Clip the skull between eyes, cut alongside the middle route of the skull and horizontal line upon the cerebellum, then open the skull to each side.

3.2.3 Lift the occipital bone with tweezers and open the spinal canal bilaterally with ophthalmic scissors. Cut off the ribs on both sides and remove the upper half of vertebrae carefully.

3.2.4 Lift the brain with curved tweezers and sever the nerves of the skull base, then dissect out the whole brain and spinal cord carefully. Post-fix the tissues in 4% paraformaldehyde for 24 h.

3.3 Preparation of tissue slices

3.3.1 Cryoprotect the brain and cervical and lumbar spinal cord in 30% sucrose solution overnight at 4 °C. Embed the tissue in optimum cutting temperature (OCT) compound and freeze fast with liquid nitrogen.

3.3.2 Cut the tissue at 25 µm using a cryostat and store the frozen sections in 0.01 M PBS at 4 °C for use.

4. Immunohistochemistry

4.1 Pretreat free-floating sections in 1% H₂O₂ for 10 min, then wash in PBS for 10 min. Incubate in a blocking solution containing 5% serum and 0.3% non-ionic detergent in PBS for 1 h.

4.2 Incubate the slices with corresponding primary antibodies overnight at 4 °C. Wash the sections in PBST (0.2% Tween 20 in PBS) for 30 min (3 times for 10 min each).

4.3 Incubate the slices with corresponding biotin-secondary antibody at room temperature for 1 h. Wash in PBST for 30 min (3 times for 10 min each).

4.4 Incubate the sections with affinity biotin peroxidase complex for 40 min, and stain with a chromogenic agent. Mount the sections onto slides and dry properly.

4.5 Soak the slides in anhydrous ethanol for 5 min and xylene for 10 min, then seal the slides with a mounting medium. Finally, image the slides with a microscope equipped with a charge-coupled device (CCD) at 100X, 200X, and 400X magnifications.

REPRESENTATIVE RESULTS:

Mice showed different degrees of transient weakness right after IT injection of AAV solution in 1% lidocaine hydrochloride due to various quality of intrathecal injection. According to the semi-quantitative 5-grade scoring system we have established, we tested the transduction patterns of AAV in mice with different degrees of lidocaine-induced limb weakness (score 0, n = 2; score 1, n = 1; score 4, n = 4; score 5, n = 3). EGFP immunostaining of spinal cords showed either no or little transduction in the lumbar spinal cord of mice scoring 0, slightly enhanced transduction in mice scoring 1, and strong and widespread transductions in mice scoring 4 or 5 (**Figure 1A**). We quantified the GFP staining intensity of those mice displaying various degrees of transient limb weakness (**Figure 1B**) and concluded that the severity of weakness after injection correlated closely with the extent of spinal cord transduction.

We further explored the detailed transduction profile of rAAVrh10 in the whole CNS, and noticed that full length of the spinal cord and wide areas of the brain were well transduced in well-injected mice which scored 4 or 5. In the brain, robust EGFP signals were detected in olfactory bulb (**Figure 2A**), dorsolateral prefrontal cortex (**Figure 2B**), dentate gyrus and CA3 zone of hippocampus (**Figures 2C and 2D**), cerebellar cortex (**Figure 2E**), and marginal areas of brainstem including facial nucleus (**Figure 2F**), choroid plexus, and ependymal epithelial cells (**Figure 2G**). However, fewer EGFP-positive cells were detected in deep regions of the brain. In the spinal cord and ventral and dorsal horns, the ventral effluent motor axons and dorsal affluent sensory axons were strongly GFP-positive. Motor neurons in the anterior horns were strongly transduced in different levels of the spinal cord (**Figures 2H-2J**). Moreover, GFP-positive neurons in the cortex including pyramidal cells were detected (**Figures 3A and 3B**). Various glial cell types including microglia, astrocytes and oligodendrocytes, were also found to be EGFP-positive (**Figures 3C-3E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Lidocaine-induced weakness extent predicts transduction efficiency. AAV with 1% lidocaine or PBS (control) was injected by direct IT injection. Mice were sacrificed and examined for GFP expression by immunohistochemistry 3 weeks later (score 0, n = 2; score 1, n = 1; score 4, n = 4; score 5, n = 3). (A) GFP staining of cervical (CSC) and lumbar spinal cord (LSC) sections is shown. (B) GFP staining intensity in both LSC and CSC was directly correlated with the degree of transient weakness. Each mark represents values (mean \pm SD) from one mouse. This figure has been adapted from a previous publication⁴.

Figure 2: Widespread transduction of rAAVrh10 in the brain and spinal cord. (A) Olfactory bulb; (B) cortex; (C) dentate gyrus⁴; and (D) CA3 of hippocampus; (E) cerebellar cortex; (F) facial

nucleus; (G) lateral ventricle; (H) cervical anterior horn⁴; (I) thoracic anterior horn⁴; and (J) lumbar anterior horn⁴. Bars represent 100 μ m.

Figure 3: Transduction of various cell types in the brain after direct intrathecal rAAVrh10 injection. (A) Pyramidal cells; (B) multipolar neuron; (C) microglial cell; (D) astrocyte; and (E) oligodendrocyte. Bars represent 100 μ m.

DISCUSSION:

Technically, there are several critical steps during the IT injection in awake mice. First, proper gesture and firm control of the mice throughout the entire operation is a prerequisite for successful delivery. Second, the most difficult point is feeling the intervertebral space with the needle tip, as it is necessary not to insert too deeply without resistance or insert forcibly under strong resistance in the case of injuring the animals or bending the needle tip. Third, although the transient paralysis due to lidocaine provides an objective indicator for IT injection quality, more practice is needed to achieve consistent and successful results.

In this report, we have developed a direct intrathecal injection method in awake mice for delivery of AAV, in which lidocaine serves as an indicator for the extent of IT injection success and as a predictor for efficiency of gene therapy. Experimental direct lumbar puncture was first used to deliver agents, especially anesthetics, to the spinal cord for analgesia and anesthesia, and it has been highly recommended in gene therapy for CNS diseases. Given the difficulty of IT injection in smaller animals like mice, we combined the two applications of direct lumbar puncture and chose local anesthetics (lidocaine, which has been used in clinics widely as an objective indicator of injection quality by evaluating transient and restorable paralysis). Additionally, we defined a standard to predict delivery efficiency of AAV through paralysis levels and confirmed this by immunostaining. We demonstrated that the well-injected animals had higher levels of rAAVrh10-EGFP transduction in the CNS in adult mice.

Compared with the previous intrathecal delivery method involving deep anesthesia of the animal and intrathecal catheterization with laminectomy^{14,15}, our current method has several advantages. First, the simple lumbar puncture procedure can be completed within a few minutes for each animal, whereas the previous procedure takes ~1 h per animal. Second, the current method does not employ anesthesia and surgery, and therefore reduces the risk of injury⁴. Third, by addition of 1% lidocaine hydrochloride to the AAV solution, we established a five-point scoring system to rank transient paralysis following the injection and proved that the degree of weakness induced by lidocaine can be used to predict the extent of CNS transduction by each injection. Our data demonstrated that the well-injected animals have high levels of rAAVrh10-EGFP transduction in the CNS of adult mice. The transduction is also widespread to a similar extent of the earlier method involving laminectomy and intrathecal catheterization. Compared with existing IT puncture methods in awake mice, we provide an objective indicator of injection quality by using lidocaine and avoid the blindness to failed injection and subsequent interference in therapeutic efficacy.

Taken together, the current intrathecal delivery containing 1% lidocaine is a promising method

in experimental therapies for CNS diseases by delivering genes or drugs in mice. Furthermore, it is a practical and convenient approach to practice IT injection in small animals such as mice.

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DISCLOSURES:

The authors have nothing to disclose.

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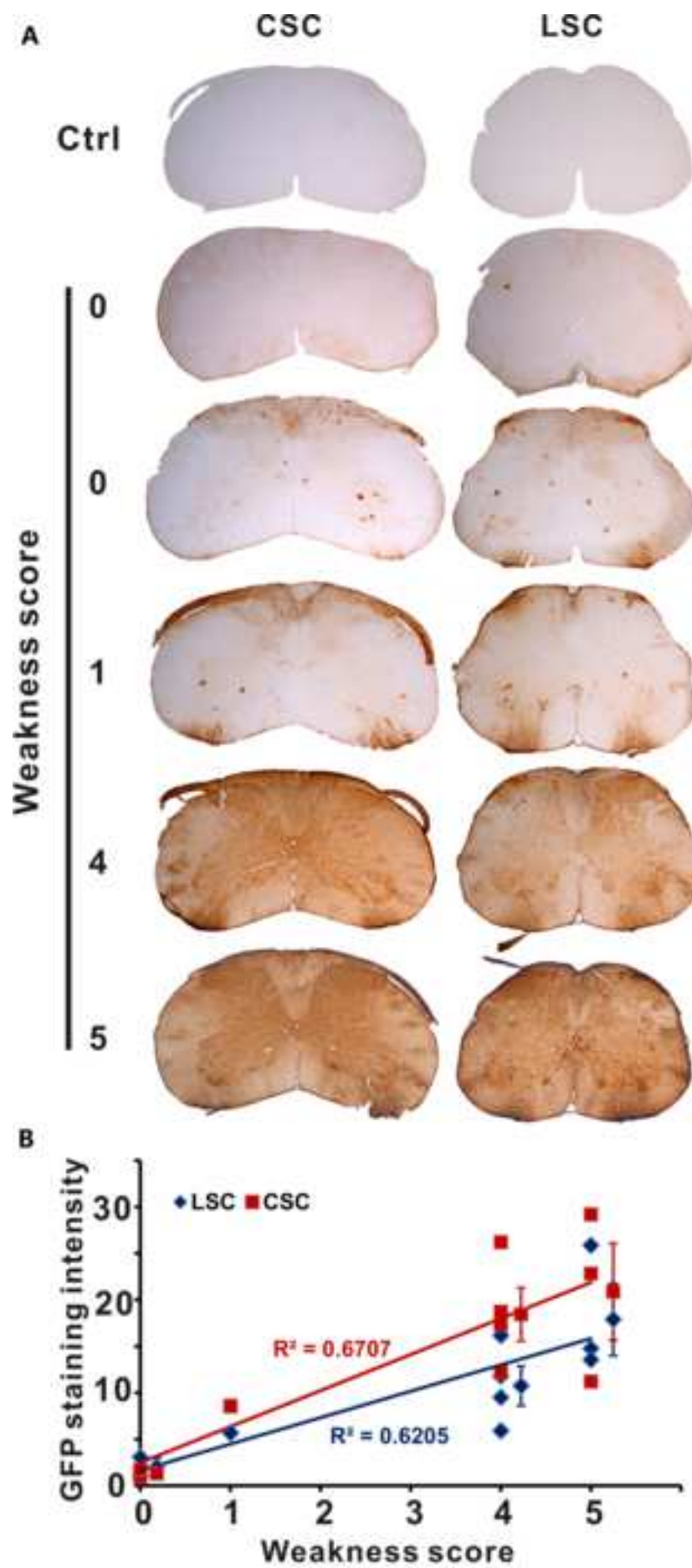
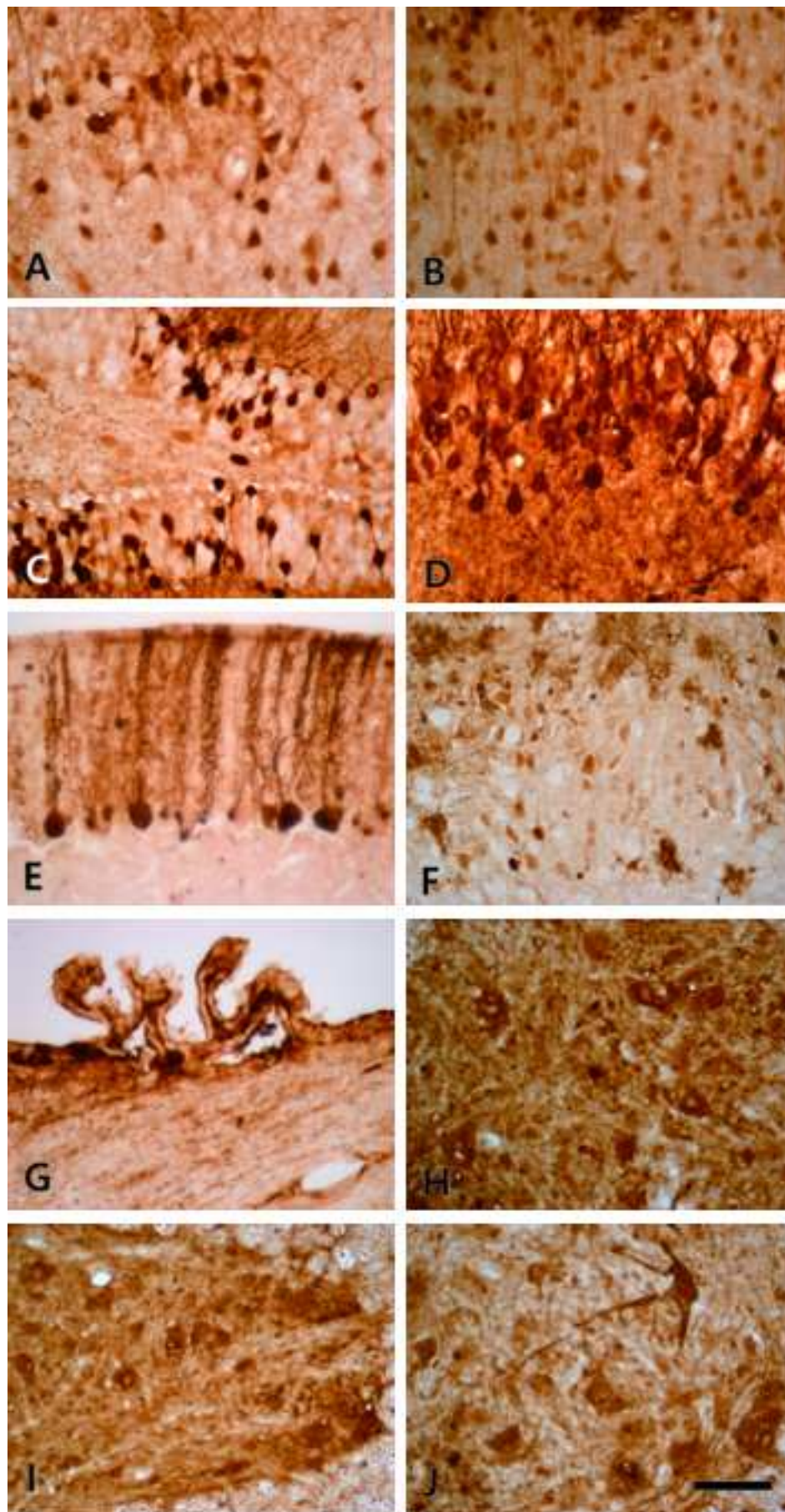


Fig. 1

**Fig. 2**

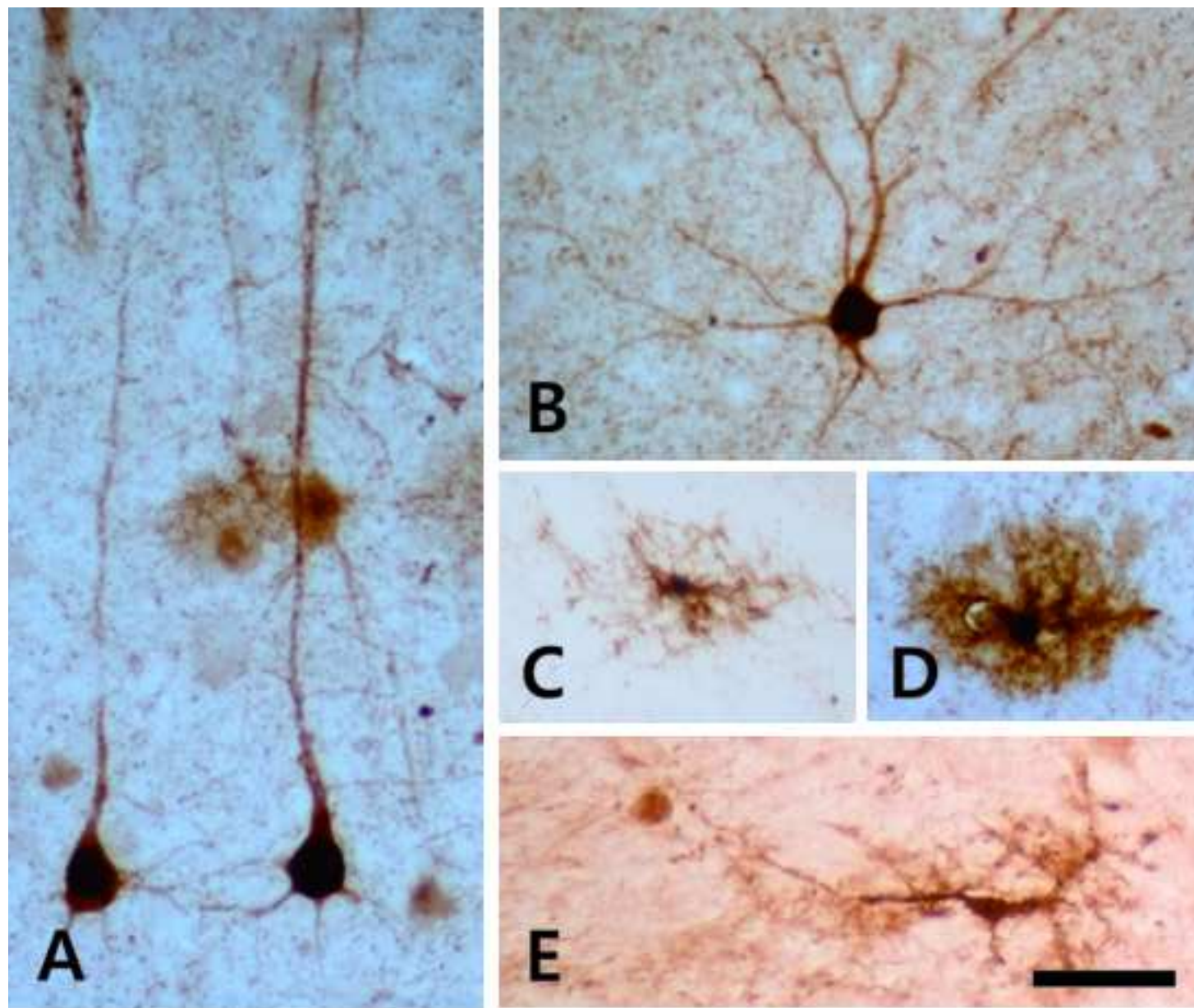


Fig. 3

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
FVB/NJ mice	Charles River Laboratories China		
Lidocaine hydrochloride monohydrate	HEOWNS	73-78-9	
AAV	Viral Vector Core of the Gene Therapy Center at University of Massachusetts Medical School		
25µL Hamilton syringe/27-30g needle	GASTIGHT	1702	
O.C.T compound	SAKURA	4583	
H ₂ O ₂	SHUI HUAN PAI	170401	
Goat serum	Solarbio	S9070	
Triton X-100	LIFE SCIENCES	T8200	
Rabbit anti-GFP	Life tech	G10362	1:333 dilution
The second antibody (goat-anti rabbit)	Jackson Immuno Research	111-005-144	1:1000 dilution
VECTASTAIN ABC REAGENT	Vector Lab	PK-6100	
ImmPACT DAB Peroxidase Substrate Kit	Vector Lab	SK-4105	
Mounting medium for fluorescence with DAPI	Vectorshield	H-1200	
NaCl	Yong Da Chemical		
NaH ₂ PO ₄ ·2H ₂ O	Yong Da Chemical		
Na ₂ HPO ₄ ·12H ₂ O	Yong Da Chemical		
Paraformaldehyde	Yong Da Chemical	307699	
Adhesion Microscope Slides	CITOGLAS	17083	25*75 mm

SUPER-SLIP MICRO-GLAS	Electro Microscopy Siences	72236-60	24*60 mm
15 ml Centrifuge tube	CORNING	430790	
96 well cell culture cluster	Coster	3599	
24 well cell culture cluster	Coster	3524	
70% Ethanol	WEN ZHI		
Gauze	Wei AN	05171112	8cm*10cm*12cm
1mL syringe	Hong Da		
Microtubes	Plasmed		
Micropipet	eppendorf		
Peppet tips	Rainin		
Centirifuge	eppendorf	5427R	
Regerator	Haier	BCD-539WT	
Filter	MILLEX GP	R4PA42342	
Pump	LongerPump	BT-100-2J/YZ1515X	
Microscope	Olympus	BX53	
Freezing-microtome	Leica	CM1520	

Title of Article: Direct intrathecal injection of recombinant adeno-associated viruses in adult mice

Author(s): Dongxiao Li, Yundu Li, Yunyun Tian, Zuoshang Xu, Yansu Guo

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