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AAV-Mediated Gene Delivery to the Enteric Nervous System by Intracolonic Injection

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Abstract

The enteric nervous system of the lower gastrointestinal tract comprises intrinsic neural circuits as well as extrinsic afferent and efferent innervation. The development of strategies for neuronal gene transfer has created new opportunities for functional analysis, circuit mapping, and neuromodulation in the enteric nervous system. Studies of AAV-mediated gene transfer to enteric neurons and dorsal root ganglion neurons (DRG) have provided proofs-of-concept for the utility of AAV vectors for genetic manipulations of the intrinsic and extrinsic components of the enteric nervous system. Here we describe a method for AAV-mediated gene transfer to enteric neurons of the descending colon as well as colon-innervating DRG neurons by injection within the intestinal wall (intracolonic injection).

Keywords

Enteric; Colon; Dorsal root ganglion; Viral vector

1 Introduction

The enteric nervous system of the lower gastrointestinal tract comprises intrinsic neural circuits as well as extrinsic afferent and efferent innervation [1]. Circuits of intrinsic sensory neurons, interneurons, and motor neurons control gastrointestinal motility and fluid movement between the intestinal lumen and body fluid compartments. The functions of enteric neural networks have been explored extensively for development of therapeutic

control of dysregulated motility. Extrinsic efferent innervation in the colon is provided by pre-enteric neurons within the spinal cord, prevertebral ganglia, and pelvic ganglia. Extrinsic primary afferent fibers supplying colon innervation reside in thoracolumbar and lumbosacral dorsal root ganglia (DRG) and transmit sensory information from the colon to the spinal cord under normal conditions and under conditions of visceral hypersensitivity. Modulation of the activity of the spinal afferent innervation of the colon through pharmacotherapy has been pursued for the management of abdominal pain and discomfort that accompany chronic gastrointestinal disorders such as irritable bowel syndrome [2, 3].

The development of strategies for neuronal gene transfer has created new opportunities for functional analysis, circuit mapping, and neuromodulation in the enteric nervous system. Adeno-associated virus (AAV) vectors have emerged as a preferred tool for gene transfer to neurons in basic research applications [4] and as promising candidates for therapeutic gene delivery, with a number of ongoing clinical trials [5]. While AAV vectors have been widely used for gene transfer in the central nervous system, applications in the peripheral nervous system have been more limited. AAV-mediated transduction of primary afferent neurons via intrathecal (i.t.) infusion [6–9], sciatic nerve injection [8], or direct intraganglionic injection [10] has been described in a number of studies. AAV vectors have also been used successfully for transduction of enteric neurons by direct injection within the intestinal wall [11] as well as by systemic [12–14], and to a lesser extent intrathecal [15], administration. Therefore, the utility of AAV vectors for genetic manipulations of the intrinsic and extrinsic components of the enteric nervous system has been demonstrated.

Here we describe a method for AAV-mediated gene transfer to enteric neurons of the descending colon as well as colon-innervating DRG neurons by injection within the intestinal wall (intracolonic injection). This route of delivery has been used extensively for injections of neuronal tracers to label colon-innervating DRG neurons [16–20] and has also been employed for transduction of enteric neurons using AAV [11]. In addition to DRG and enteric neurons, limited transduction of nodose ganglion neurons is possible, particularly if the vector is delivered to proximal colon. Gene transfer to extrinsic efferent neurons in prevertebral and pelvic ganglia is also possible, but has not been evaluated. Transduction of pre-enteric neurons in medulla and spinal cord was not observed [11].

Several factors need to be considered when planning intracolonic AAV delivery, including AAV serotype and titer, the promoter used in the viral construct, and the injection volume. AAV serotypes that have been tested in the enteric nervous system include AAV1, 2, 5, 6, 8, and 9 [11, 12]. In adult subjects, neuronal tropism was observed with all serotypes, while AAV6 also transduced enteric glia [11]. Transduction of smooth muscle cells was negligible. The highest transduction efficiency was seen with AAV6 and AAV9 in both the myenteric and submucosal plexus of the enteric nervous system. Preferential targeting of cholinergic enteric neurons by AAV9 has been suggested [12], but differential transduction of subtypes of enteric neurons has not been explored comprehensively. In our experiments, we have also observed transduction of enteric neurons with AAV9 vectors (Fig. 1a), and we consistently observe AAV9-mediated gene transfer to colon-innervating sensory neurons (Fig. 1b). Viral titers of $\sim 10^{12}$ vector genomes (vg)/mL transduce approximately 30% of enteric neurons, while titers of $\sim 10^{13}$ vg/mL transduce over 70% of neurons [11]. Although to date only

constructs with ubiquitous promoters have been used in the enteric nervous system, non-ubiquitous promoters such as the promoter for the choline acetyltransferase gene may be employed to achieve preferential targeting of subtypes of enteric neurons. Finally, injection volumes of 2–5 μL have been used for intracolonic injections. The injection volume affects the spread of the virus within the enteric nervous system; however direct comparison of different injection volumes has not been performed.

2 Materials

1. Male and female CD-1 or C57/Bl6 mice, 4–8 weeks old, weighing between 17 and 40 g. Mice younger than 3.5 weeks or weighing less than 15 g are challenging to inject due to their small size.
2. AAV vector. A titer of 10^{12} vg/mL or higher is recommended (*see* Note 1).
3. Microcentrifuge.
4. Isoflurane.
5. 70% ethanol (v/v).
6. Betadine (povidone-iodine).
7. Analgesic agent, such as Meloxicam.
8. Artificial tears or ocular lubricant.
9. Stereoscope or dissecting microscope.
10. Vaporizer for delivery of anesthetic gas.
11. Scavenger system for anesthetic gas.
12. Heating pads.
13. Oxygen tank.
14. Glass bead sterilizer or chemical sterilizer.
15. Anesthesia nose cone.
16. Sterile cotton-tipped applicator.
17. Sterile cotton gauze, or alternative method to control bleeding.
18. Sterile saline.
19. Nair hair removal cream.
20. 4–0 violet monofilament absorbable suture (Ethicon).
21. 10 μL Hamilton gastight removable needle syringe.
22. 30G, 0.5'' Hamilton needle, point style 4.
23. 3M™ Vetbond™ tissue adhesive.

¹ AAV vectors should be stored in small aliquots at -80°C to avoid freeze/thaw cycles.

24. 0.5 mL syringe for dispensing Vetbond.
25. 0.5 mL syringe for administration of analgesics.
26. Scalpel holder and blades.
27. 2 pairs curved blunt forceps.
28. Spring scissors.
29. Self-restraining cross action retractor with 4" blunt prongs, such as Miltex® 11–16.
30. 2 pairs of forceps for wound closure.
31. Needle holder with suture cutter.
32. Parafilm.
33. Micropipette and micropipette tips.
34. Paper towels or other means to elevate and stabilize the animal in the nose cone.
35. Recovery chamber with heating pad.
36. Tattoo ink to mark injection site.

3 Methods

3.1 Setup and Anesthesia

1. Sterilize surgical tools using an autoclave, glass bead sterilizer, or chemical sterilizer.
2. Autoclave Hamilton syringe before and after vector application to avoid cross contamination if several vectors are injected with the same syringe.
3. Position nose cone over the heating pad.
4. Administer volatile anesthesia (3.5–4% Isoflurane) using an induction chamber. Once fully anesthetized, place the animal in a nose cone secured in place over a heating pad.
5. Confirm depth of anesthesia by checking paw withdrawal reflex with toe pinch.
6. Once desired depth of anesthesia is achieved, apply ocular lubricant.
7. Place the mouse in dorsal recumbency.
8. Apply hair removal cream for 2–3 min.
9. Wipe the hair removal cream with deionized or sterile water.
10. Clean the abdominal surface with 70% ethanol.
11. Alternate betadine and alcohol wipes three times.

3.2 Surgical Procedure

1. Thaw an amount of AAV sufficient for all injections to be performed during the day. Spin the tubes with vector in a microcentrifuge to ensure that fluid is not retained in the tube cap. Store on ice until use.
2. After confirming deep anesthesia, make a midline incision on the lower abdomen under a microscope to expose the skin overlaying the peritoneum. Start the incision below the sternum and continue posteriorly until the preputial gland is exposed.
3. Using spring scissors, open the peritoneal cavity by making a midline incision along the linea alba starting below the xiphoid process and extending ~5 cm caudally until the preputial gland. Do not cut or damage the preputial gland.
4. Using blunt curved forceps, move midline organs laterally to visualize the distal colon.
5. Insert retractor into the abdominal cavity to gently move the intestines and surrounding organs to provide a clear view of and access to the descending colon. Ensure that the teeth of the retractor do not press upon the organs directly as this may cause organ damage (*see* Note 2).
6. Pipette 4 μ L of AAV onto a piece of parafilm and withdraw into a 10 μ L Hamilton syringe. This ensures an accurate volume of vector administration (*see* Note 3).
7. With a small paint brush, brush tattoo ink onto the tip of the needle to mark the injection site. Marked injection sites are shown in Fig. 1c.
8. With curved blunt forceps, gently hold the distal portion of the colon and tug cranially to gain access to the caudal portion of the distal colon (2 cm from the anal verge, as close as possible to the pelvic bone).
9. While holding onto a portion of the colon with blunt curved forceps, insert the needle of the Hamilton syringe aborally below the serosa at a 30–45 degree angle, then tilt the syringe to ~20 degrees. Tunnel the needle 2–3 mm subserosally and inject the vector slowly over 30–45 s. If the vector is correctly injected, a slight bulge can be observed at the injection site (*see* Note 4).
10. Hold the needle in place for at least 1 min and then slowly retract the needle. Gently wipe the injection site with a cotton swab to remove any excess or leaked vector and flush the abdominal cavity with sterile saline.
11. Close the peritoneum with 4–0 absorbable suture.

². Access to the distal colon is similar in males and females. Males are slightly larger than their age matched female littermates; however, in females, the distal colon can be easily visualized by retracting the uterus.

³. It is very difficult to draw small volumes into a Hamilton syringe from an Eppendorf tube. In addition, the tip of the needle can be bumped against the tube, compromising its sharpness. Once the desired volume is pipetted on parafilm, the bevel of the needle can be submerged in the fluid, which is then easily drawn into the syringe. The parafilm should be wiped with 70% ethanol before pipetting the vector onto it to prevent contamination of the injectate.

⁴. The absence of a bulge after vector injection suggests that the vector leaked out or was injected deep in the colon wall or in the lumen.

12. To close overlying skin, lift the skin on one side of the incision and dispense Vetbond. Quickly bring together the two edges of skin to appose the overlying skin. Grab and hold the apposed skin with blunt forceps for 15–30 s, then pull on the adjacent skin to test for successful adhesion before transferring the mouse to the recovery chamber.

3.3 Recovery and Postoperative Monitoring

1. Subcutaneously administer 2 mg/kg Meloxicam or an alternative analgesic in the back of the neck (scruff).
2. Place the animal in a recovery chamber with heating pad, and monitor until the animal has recovered and is mobile and alert. If the animal looks scruffy or struggles to recover from anesthesia, inject 400–500 μ L of sterile saline (s.c.) and continue monitoring.
3. Return the mouse to a clean cage. Administer analgesic for 3 days postoperatively and monitor the incision site, urination, defecation, food intake, and general activity (*see* Note 5).

3.4 Representative Results

We have used this protocol to inject AAV9 vectors carrying various constructs. After injections of AAV9-CAG-tdTomato (obtained from the Vector Core at the University of North Carolina), we can observe tdTomato expression in colon enteric neurons as well as colon-innervating dorsal root ganglia (DRG) neurons 3–4 weeks after injections. Representative images of tdTomato fluorescence are shown in Fig. 1a, b (*see* Note 6).

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⁵If the abdominal skin incision opens in the days following the surgery, reclose the overlying skin with Vetbond under anesthesia and in an aseptic environment.

⁶This procedure can be incorporated in an intersectional targeting strategy where a vector carrying Cre-recombinase and a vector carrying a Cre-dependent transgene are delivered via distinct but converging routes. For example, intracolonic delivery combined with intrathecal or intraganglionic delivery would result in selective expression of the Cre-dependent transgene in colon-innervating DRG neurons. In this case, it is recommended that the Cre-dependent vector be delivered via the route that is expected to drive the most efficient transduction of the target cells. Alternatively, injections of the two viruses at different locations within the colon could potentially transduce selectively ascending or descending enteric neurons. Injection volumes of 1 μ L or less are recommended for this approach, which would be facilitated by an infusion system such as the Pump 11 Pico Plus Elite Dual (Harvard Apparatus). If two vectors of the same serotype are to be used, they must be injected on the same day. The initial exposure to AAV may induce a response that prevents transduction with the second AAV vector.

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Fig. 1. Representative results of intracolonic AAV delivery. **(a, b)** Representative images of tdTomato-expressing neurons in the myenteric plexus of colon **(a)** and in colon-innervating sensory neurons **(b)**. Scale bars: 200 μ m. **(c)** Colon dissected from AAV9-injected mice. Injection sites are marked in green. In all cases, there was a single injection. The proximal side is on the right