# Adeno-Associated Viral Vector Delivery to the Enteric Nervous System: A Review

**Sara E. Gombash**
Department of Neuroscience, The Ohio State University, Columbus, Ohio 43210, USA.
**Email:** sara.gombashlampe@osumc.edu

**Abstract**
Gene therapy to the gastrointestinal tract has remarkable potential for treating gastrointestinal disorders that currently lack effective treatments. Adeno-associated viral vectors (AAVs) have been extensively applied to the central nervous system, and have repeatedly demonstrated safety and efficacy in animal models. The enteric nervous system (ENS) represents a vast collection of neurons and glial cells that may also be subject to treatment by AAV, however little work has been conducted on AAV delivery to the ENS. Challenges for gastrointestinal gene therapy include identifying gene targets, optimizing gene delivery, and target cell selection. Researchers are now beginning to tackle the later of the two challenges with AAV, and the same AAV technology can be used to identify novel gene targets in the future. Continued efforts to understand AAV delivery and improve vector design are essential for therapeutic development. This review summarizes the current knowledge about AAV delivery to the ENS.

**Keywords:** gene therapy, enteric nervous system, AAV, gastrointestinal system, myenteric

## Introduction

The enteric nervous system (ENS) is the innate nervous system in the gastrointestinal (GI) tract. The ENS is connected to and receives input from the central nervous system (CNS) but maintains its own neuronal circuitry and can perform without CNS input giving it the nickname the "little brain in the gut" (Wood, 2011). The ENS consists of an estimated 100 million neurons and extends through the entirety of the GI tract. Generally, neurons lie in ganglionated plexuses called the submucosal (SMP) and myenteric (MP or Auerbach Plexus) nerve plexuses. The SMP lies under the mucosal layer closest to the lumen of the gut and the MP lies between the circular and longitudinal smooth muscle layers. In large mammals and humans, the SMP is subdivided into multiple layers. Specifically, in the human ENS, the SMP is subdivided into three separate plexuses; the Meissner plexus lies closest to the mucosa, the Schabadaseh plexus is closest to the circular smooth muscle, and an intermediate plexus lies in between (Timmermans et al., 2001, Schemann and Neunlist, 2004). Each is made of a collection of neurons, including excitatory and inhibitory motor, sensory, and interneurons, as well as separate classes of enteric glia. Discussion of each cell type and function are beyond the scope of this article (for review see Furness, 2000, Gulbransen and Sharkey, 2012).

Enteric neurons and glia control and coordinate sensation, secretion, nutrient and fluid absorption and peristalsis. Disruption of proper ENS function results in gastrointestinal (GI) symptoms. Sources of ENS disruption include: 1) improper ENS development as in Hirschsprung's Disease (Amiel and Lyonnet, 2001), 2) enteric cell degeneration such as in ageing (Camilleri et al., 2008, Saffrey, 2013), degenerative neuropathies and chronic intestinal pseudo-obstruction (Hall and Wiley, 1998, Antonucci et al., 2008), and 3) inflammation as occurs in Crohn's disease, ulcerative colitis, Irritable Bowel Syndrome, Inflammatory Bowel Disease (Neunlist et al., 2003a, Villanacci et al., 2008, Venkataramana et al., 2015) or following bacterial infection such as *Clostridium difficile* (Xia et al., 2000, Neunlist et al., 2003b) or *Shigella flexneri* (Coron et al., 2009) infection. Furthermore, recent reports have established a co-morbidity of GI symptoms in CNS neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's

diseases (Andrich et al., 2009, van der Burg et al., 2011, Wu et al., 2011, Paillusson et al., 2012). Co-morbidity of GI symptoms may suggest damage to ENS neurons and/or loss of CNS regulatory control. GI dysfunction manifests as symptoms of constipation, diarrhea, vomiting, gastroesophageal reflux, delayed gastric emptying, and if severe and reoccurring can result in malnutrition and death. Currently only symptomatic treatments exist for ENS dysfunction in patients with GI symptomatology. Therapeutic progress has been hindered by a lack of available animal models of GI disease. Animal models largely consist of transgenic rodents and animals given toxic agents, therefore not selectively targeting the ENS or accurately mimicking human disease states.

Recombinant adeno-associated viral vectors (rAAV or AAV) are broadly used for gene delivery in both research and clinical applications (Mueller and Flotte, 2008, Samulski and Muzyczka, 2014). AAVs are favored for use because they are non-pathogenic, provide long-term transgene expression, produce minimal host immune response, and infect both dividing and quiescent cells (Flotte and Carter, 1995, Daya and Berns, 2008). Furthermore, elements of AAVs such as serotype and promoter sequence can be altered to direct gene expression to specific cell and tissue types (Wu et al., 2006, Samulski and Muzyczka, 2014). The ability of AAVs to deliver genes to cells in the CNS has been intensely studied in animal models (for review see Murlidharan et al., 2014), and have already been utilized in clinical trials (Samulski and Muzyczka, 2014). AAV delivery has produced efficient and robust gene expression in neuronal and glial cells of the CNS, but minimal work has been conducted to examine AAV gene delivery to the enteric nervous system (ENS).

Successful therapeutic application of AAV mediated gene therapy in the ENS is dependent on viral delivery, specific cell targeting, and identification of therapeutic genes for GI disease. Recent studies have made significant headway on our understanding of AAV delivery to and transduction of cells in the ENS. This review will provide a summary of the research conducted on AAV delivery and importance of AAV design in ENS gene transfer thus far.

**Administration Routes to the ENS**

Routes of AAV administration to the ENS determine both the efficacy and safety of potential gene therapies. Oral, rectal, direct injection and intravenous administration have been tested for AAV delivery to enteric cells. Studies using each delivery method are outlined in **Table 1**.

*Oral and Rectal Administration*

A majority of studies utilizing oral gavage or rectal enema to deliver AAV to the gut aimed to transduce cells within the gut mucosa affected in inflammatory GI disorders (Shao et al., 2006, Polyak et al., 2008, Polyak et al., 2012). Consequently little has been reported on AAV delivery to the ENS using these delivery methods. Oral administration is an attractive option for AAV delivery because it is non-invasive, can potentially transduce the entire length of the GI tract, and can elevate serum levels of a desired protein. Rectal administration by enema is also relatively non-invasive and is advantageous if only colonic transduction is desired. However both routes of delivery are particularly challenging for ENS transduction due to the thick epithelial barrier, acidic pH of the stomach, and digestive mucous and other fluids limiting the stability of AAV particles and accessibility to the deeply embedded ENS cells.

Prior rodent studies have reported transduction of cells in the epithelia and lamina propria following oral gavage of AAVs, but do not report transduction in the ENS (During et al., 1998, During et al., 2000, Shao et al., 2006, Polyak et al., 2008, Polyak et al., 2012). Importantly, the primary purpose of some of these studies was to transduce cells in superficial layers of the gut, therefore transgene expression in ENS cells was not examined. During et al. was the first to report that AAV gavage by orogastric tube transduced lamina proprina and epithelial cells

**Table 1** Summary of studies delivering AAV to the gastrointestinal tract

| Reference | AAV Serotype(s) Used | SS or SC | Promoter | Transgene | Delivery Site | Species | Age at Delivery | Dose | Outcome measure | Transgene Present in GI Tissue | Transgene Present Specifically in ENS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Oral or Enema*** | | | | | | | | | | | |
| During et al. (1998) | AAV2/2 | ssAAV | CMV | B-galactosidase | Orogastric tube | Rat | Adult (16 weeks) | 1.2E+08 | RT-PCR | Yes - transgene in lamina propria and epithelial cells | Not Reported |
| During et al. (2000) | AAV2/2 | ssAAV | CMV | B-galactosidase | Orogastric tube | Rat | Adult | 1.0E+09 | RT-PCR, IHC | Yes - transgene in lamina propria and epithelial cells | Not reported |
| Shao et al. (2006) | AAV2/2 | ssAAV | CMV | B-galactosidase | Oral Gavage | Mouse | Adult (10-12 wks) | 1.8E+10 | RT-PCR | Yes | Not reported |
| Polyak et al. (2008) | AAV2/1, 2, 5 | ssAAV | CB/CMV | GFP | Oral Gavage | Mouse | Adult (5 wks) | 1.0E+11 | RT-PCR, IHC | Yes - expression low | Not reported |
| | AAV2/1, 2, 5 | ssAAV | CB/CMV | GFP | Enema | Mouse | Adult (5 wks) | 1.0E+11 | RT-PCR, IHC | Yes - expression low in epithelia | Not reported |
| Polyak et al. (2010) | AAV2/2-8, AAV2/ rh10 | scAAV | CB/CMV | GFP | Small Bowel Gavage | Mouse | Adult (6-8 wks) | 5.0E+10 | RT-PCR, IHC | Low to none | Not reported |
| | AAV2/2-8, AAV2/ rh10 | scAAV | CB/CMV | GFP | Enema | Mouse | Adult (6-8 wks) | 5.0E+10 | RT-PCR, IHC | Low to none | Not reported |
| ***Direct Injection*** | | | | | | | | | | | |
| Benksey et al. (2014) | AAV1, AAV2, AAV5, AAV6, AAV8, AAV9, AAV2-Y444F, AAV2-tripleY-F, AAV2-tripleY-F+T-V, AAV8-Y733F, AAV8-doubleY-F+T-V | scAAV | CB/CMV | GFP | Colon Wall | Rat | Adult | 1.3E+12 x 6 injections | RT-PCR, IHC | Yes - expression serotype dependent | Yes - myenteric and submucosal neurons |
| | AAV9 | scAAV | CB/CMV | GFP | Colon Wall | Mouse | Adult | 1.2E+13 x 6 injections | IHC | Yes | Yes - myenteric and submucosal neurons |
| ***Intravenous Injection*** | | | | | | | | | | | |
| Grimm et al. (2008) | AAV2, AAV8, AAV9, AAVDJ, AAVDJ/8, AAV2/8 | scAAV | CMV | Human Factor IX | Tail Vein | Mouse | Adult (6-8 wks) | 5.0E+11, 1E+12 or 7.0E+12 | PCR | Yes - serotype dependent | Not Reported |
| Polyak et al. (2008) | AAV2/1, 2, 5 | ssAAV | CB/CMV | GFP | SMA | Mouse | Adult (5 wks) | 1.0E+11 | RT-PCR, IHC | Yes - serotype dependent | Not reported |
| Zinvarelli et al. (2008) | AAV2/1-9 | ssAAV | CMV | Luciferase | Tail Vein | Mouse | Adult (8-10 wks) | 1.0E+10 | Bioluminscent imaging | Yes - serotype dependent | Not reported |
| Polyak et al. (2010) | AAV2/2-8, AAV2/rh10 | scAAV | CB/CMV | GFP | SMA | Mouse | Adult (6-8 wks) | 5.0E+10 | RT-PCR, IHC | Yes - serotype dependent, epithelial transduction | Not reported |
| Rahim et al. (2011) | AAV2/9 | scAAV, ssAAV | CMV | GFP | Vitelline Vessel | Mouse | Embryonic | 2.0E+11 | IHC | Yes | Yes - in myenteric ganglia |
| | AAV2/9 | scAAV, ssAAV | CMV | GFP | Temporal Face Vein | Mouse | Neonate | 4.0E+11 | IHC | Yes | Yes - in myenteric ganglia |
| Bevan et al. (2011) | AAV2/9 | scAAV | CB/CMV | GFP | Temporal Face Vein | Cytomologus Macaque | Neonate and Young Adult (P1, 30 90) | 1-3.0E+14 | IHC | Yes | Yes |
| Hu et al. (2012) | AAV2/1-9, AAV2/rh10 | ssAAV | CB/CMV | Luciferase | Temporal Face Vein | Mouse | Neonate | 2.0E+10 | Bioluminscent imaging | Yes - serotype dependent | Not Reported |
| Schuster et al. (2014) | AAV2/9 | ssAAV | CB/CMV | GFP | Tail Vein | Mouse | Adult (6-8 wks) | 3.3E+11 | IHC | Yes | Yes |
| Gombash et al. (2014) | AAV2/8, AAV2/9 | scAAV | CB/CMV | GFP | Temporal Face Vein | Mouse | Neonate | 1.0E+11 or 5.0E+10 | IHC | Yes | Yes - AAV2/9 myenteric neurons, AAV2/8 myenteric neurons and glia |
| | AAV2/9 | scAAV | CB/CMV | GFP | Tail Vein | Mouse | Young Adult | 2.0E+12 | IHC | Yes | Yes - transduction of myenteric neurons |
| | AAV2/9 | ssAAV | GFAP | GFP | Temporal Face Vein | Mouse | Neonate | 1.1E+11 | IHC | Yes | Yes - enteric glia transduction |
| ***Other Injection Methods*** | | | | | | | | | | | |
| Polyak et al. (2008) | AAV2/1, 2, 5 | ssAAV | CB/CMV | GFP | Intraperotineal | Mouse | Adult (5 wks) | 1.0E+11 | RT-PCR, IHC | Yes - smooth muscle only | Not reported |
| Schuster et al. (2013) | AAV2/8 | ssAAV | CB/CMV | GFP | Intrathecal | Mouse | Adult (20-25g) | 6.0-7.0E+10 | IHC | Yes | Yes - GFP in fibers only |
| Schuster et al. (2014) | AAV2/9 | ssAAV | CB/CMV | GFP | Intrathecal | Mouse | Adult (6-8 wks) | 3.3E+11 | IHC | Yes | Yes - GFP in fibers and cell bodies |

efficiently (During et al., 1998, During et al., 2000). Shao et al. reported that AAV stability could be enhanced after oral administration by neutralizing gastric acid with sodium bicarbonate and inhibiting protease activity, however AAV transduction remained low in the mucosal lining and was absent in the ENS (Shao et al., 2006). Administration of mucolytic agents such as N-acetyl-L-cysteine (NAC) and pilocarpine do not enhance AAV transduction in mice (Polyak et al., 2012). To date, no studies have reported successful ENS transduction following oral administration.

Similarly, Polyak et al. reported that rectal enema of AAV produced no significant transduction of the mouse GI tract (Polyak et al., 2012). AAV transcytosis across the epithelial barrier in a cell and serotype specific manner has previously been reported when tested *in vitro* model systems of epithelia, none of which were specifically gut epithelial cells (Di Pasquale and Chiorini, 2006). *In vivo* evidence thus far suggests that AAVs are unable to effectively penetrate the mucosal lining of the GI tract to transduce underlying enteric neurons and glia. Together, these studies suggest that oral or rectal delivery may be ideal for inflammatory GI disorders in which only cells in the mucosal layer are the primary targets.

*Direct Injection*

Direct injection of AAVs into the gut to transduce enteric cells is an efficacious method of AAV administration for localized, targeted gene delivery. Direct injection limits the biodistribution of vectors and significantly reduces off-target cell/organ transduction, however, is highly invasive and requires surgery for exposure of the GI wall. Nishi et al., was the first to investigate direct injection by performing intragastric injections into the smooth muscle or serosal injections of naked plasmid DNA expressing luciferase (pCMV-luciferase) in mice (Nishi et al., 2008a, b). Gene expression was measured by luciferase assay, and expression was detected in the stomach only after serosal injection. Although this study utilized injection of plasmid DNA rather than complete AAVs, it was among the first to document that direct injection produced gene expression confined to a single GI region.

To date, only a single study has thoroughly characterized ENS transduction following direct AAV injection. Benskey et al., reported efficient ENS transduction following the injection of multiple AAV serotypes expressing green fluorescent protein (GFP) directly into the wall of the rat descending colon (Benskey et al., 2015). GFP positive neurons and/or glial cells were observed in both the SMP and the MP. Evidence of transduction was observed multiple millimeters away from the transduction site and transduction area is increased by injecting in multiple sites along the colon wall. Transduction efficiencies and cell tropism were dependent on AAV serotype and ranged from 2.7-31.0% or 2.7-36.4% of neurons in the MP and SMP, respectively. Benskey et al. also reports no evidence of viral spread outside the gut into other organs or in circulation (Benskey et al., 2015). Thus far, direct injection is the only method of efficient ENS gene delivery able to confine transgene expression to a single area within the GI tract.

*Systemic Injection*

Systemic injection of AAVs has consistently and repeatedly transduced the ENS in multiple animal species. The discovery that AAV9 was able cross the blood brain barrier and transduce CNS tissues following intravenous (IV) injection in rodents popularized this delivery method and inspired a multitude of studies investigating AAV tissue distribution after systemic administration (Grimm et al., 2008, Zincarelli et al., 2008, Duque et al., 2009, Foust and Kaspar, 2009, Foust et al., 2009, Hu et al., 2010, Rahim et al., 2011, Schuster et al., 2014). Consequently, several studies have reported transgene expression in the ENS following intravenous injection into the vitelline vessels, superficial temporal face vein, superior mesenteric artery, and tail vein of rodents (Grimm et al., 2008, Polyak et al., 2008, Hu et

al., 2010, Rahim et al., 2011, Polyak et al., 2012, Schuster et al., 2013, Gombash et al., 2014, Schuster et al., 2014). Additionally, Bevan et al., reported GFP transgene expression in the ENS of cynomolgus macaques following injection of AAV9 demonstrating the feasibility of IV delivery in a large animal model (Bevan et al., 2011). Systemic injection of AAV is a favored delivery method for research because the technique is easily mastered by research personnel and injection is fast and relatively non-invasive, except in cases of superior mesenteric artery injection for which a laparotomy is required (Porvasnik et al., 2010). Systemic administration is clinically advantageous because it is relatively non-invasive and IV placement is already common in clinical practice. IV AAV delivery is currently being used to deliver systemic gene therapy in a clinical trial for spinal muscular atrophy (Mendell). Disadvantages include having to inject large volumes of virus to achieve optimal dosing in large animal models or in patients and global AAV distribution throughout the body, however in specific disease cases, systemic transgene expression is acceptable and even desired.

A single published study has characterized AAV transduction efficiency and tropism in the ENS of multiple types of AAVs following systemic administration (Gombash et al., 2014). By injecting AAV expressing GFP into the temporal face vein of neonatal mice or the tail vein of juvenile mice, our group demonstrated robust AAV transduction in enteric cells through the entire length of the GI tract. GFP positive neurons were quantified in the stomach, duodenum, jejunum, ileum, cecum and colon of neonatal and juvenile injected mice. In the neonatally injected MP, transduction percentages ranged from approximately 25-43% of enteric neurons. Percentages ranged from approximately 38-57% in juvenile mice and transduction was dose dependent. Furthermore, these studies have been replicated in our laboratory in neonatal guinea pigs, in which significant transduction of the SMP and MP has been observed (unpublished data).

*Intraperitoneal and Intrathecal Injection*

Both intraperitoneal (IP) and intrathecal injections of AAVs have been used to transduce GI tissues. Polyak et al. reported that IP injection results in AAV transduction of smooth muscle of the gut, but saw no additional transgene expression in other cell layers (Polyak et al., 2008). ENS specific transduction was not examined in that study. In studies by Schuster et al., AAV8 or AAV9 expressing GFP was intrathecally injected into adult mice (Schuster et al., 2013, Schuster et al., 2014). AAV8 injection resulted in transgene expression to extrinsic spinal afferents projecting to the colon (Schuster et al., 2013). Alternatively, AAV9 injection resulted in transgene expression in enteric cell bodies and fibers (Schuster et al., 2014). Our lab has observed GFP transgene expression in both enteric neurons and their projection after intrathecal AAV9 injection into neonatal mice (Gombash et al., 2015).

**AAV cell tropism in the ENS: Controlling transgene expression through vector design**

Viral serotype, promoter sequence, and enhancer elements can be manipulated in a number of ways to control transgene expression *in vivo* (Burger et al., 2005, Murlidharan et al., 2014). Cell tropism is dependent on AAV serotype and the existence of specific cell surface receptors and co-receptors for infectivity, however attachment receptors have only been identified for some AAV serotypes. Pseudotyping AAVs, in which the genome of one inverted terminal repeat (ITR) from one serotype is packaged in a different serotype capsid, modifies cell tropism. Prior studies have demonstrated that AAV serotype/pseudotype highly influences the type of cell that will be infected in CNS tissue (Burger et al., 2004, McCown, 2011, Aschauer et al., 2013, Chakrabarty et al., 2013). Cell specific expression can be further refined through alterations of the promoter element in the AAV DNA viral cassette. The promoter sequence

drives transgene expression in a cell-specific manner and influences expression levels (for review see Fitzsimons et al., 2002). Enhancer elements, such as a woodchuck promoter response element (WPRE), can be used to increase transgene production. Furthermore, self-complementary AAVs (scAAV), by circumventing the rate-limiting step of second strand DNA synthesis, can be used to drive higher levels of transgene expression more quickly then single-stranded AAV (ssAAV) (Gray and Zolotukhin, 2011).

*Impact of Serotype on Transduction*

AAV types used in studies examining ENS transduction are listed in **Table 1**. Two recent studies have characterized variations in cell tropism following delivery of multiple AAV serotypes to the ENS (Gombash et al., 2014, Benskey et al., 2015). Recently, Benskey et al. compared and characterized ENS transduction following direct injection of wild type AAV serotypes 1, 2, 5, 6, 8, 9 and tyrosine mutant vectors AAV2-Y444F, AAV2-tripleY-F, AAV2-tripleY-F+T-V, AAV8-Y733F, and AAV8-doubleY-F+T-V capsid mutants into the wall of the rat colon (Benskey et al., 2015). All vectors expressed GFP under the control of the chicken-β-actin/cytomegalovirus promoter (CB/CMV). They report that direct injections of AAV1 and 2 result in low levels of transduction determined by GFP positive cell count in the colon MP and SMP, AAV5 and 8 transduced intermediate numbers of cells, and AAV6 and 9 resulted in robust transduction, demonstrating the impact of serotype on ENS transduction efficacy. GFP expression was confined to enteric neurons with AAV2 and AAV9 injection. Both neurons and enteric glia were transduced by AAV1, 5, 6, and 8. Neuronal transduction was favored by all serotypes with the exception of AAV6, in which neuronal and glial transduction was equal. Benskey et al. then repeated direct injections of AAV9 into the colon walls of mice to demonstrate the feasibility of AAV transduction in the gut across species.

Our group was the first to examine ENS cell transduction for pseudotyped AAV2/1, 2/5, 2/6, 2/8 and 2/9 administered by intravenous injection (Gombash et al., 2014). Our self-complementary AAVs were constructed with the CB/CMV promoter, expressed GFP, and were injected in neonatal mice on P1 at equivalent doses between groups. GFP expression was observed in brain and spinal cord tissues of AAV2/5, AAV2/6, AAV2/1, AAV2/8 and AAV2/9, in order of increasing expression. In the ENS, AAV2/1, 2/5, and 2/6 produced little to no GFP expression. AAV8 and AAV9 robustly transduced enteric MP and SMP (Gombash et al., 2014, unpublished data). GFP expression mediated by AAV8 infection was observed primarily in enteric neurons and in small numbers of enteric glia in the MP. Prior studies utilizing intravenous injections of AAV9 show that when administered to neonatal mice, AAV9 transduces primarily neurons in the CNS. Alternatively, AAV9 injected into adult animals via tail vein preferentially infects CNS astrocytes. In contrast to CNS data, in the ENS, the age-dependent transduction bias was absent and neurons were exclusively transduced.

Further analysis completed with immunohistochemistry revealed that AAV8 and AAV9 infected different classes of enteric motor neurons. Ongoing studies in our laboratory show that IV injection of AAV results in transduction of enteric neurons in guinea pigs and cynomolgus macaques, further demonstrating the feasibility of this approach in larger species (unpublished data). Importantly, the AAV serotype used in larger animals transduced different classes of enteric cells demonstrating species dependent variances in infectivity (unpublished data).

*AAV Promoters and Cell Specificity*

Many studies have utilized varying promoters to drive cell-specific expression in the CNS. The CB/CMV promoter has been used by a majority of GI transduction studies due to its known robust expression. In order to enhance enteric

glial cell transduction in the ENS, we altered our vector to have a glial fibrillary acid protein (GFAP) promoter within our AAV2/9 construct. When intravenously injected into neonatal mice, GFP transgene expression was primarily detected in enteric glia (Gombash et al., 2014). This data demonstrates the ability of AAVs to be targeted to specific cell populations. Further vector development will allow for expression refined to targeted populations of enteric motor neurons, interneurons, and Interstitial Cells of Cajal.

**Discussion**

Gene therapy to the ENS has represented an immense challenge requiring significant advances in knowledge of target genes, target cells, suitable vector systems and methods of targeted delivery for therapeutic gene expression. Collectively, the studies described in this review show promising preclinical data that demonstrate the feasibility of AAV delivery to the ENS. Changes in AAV vector construction have allowed for increased specificity in cell targeting, and studies are underway in our laboratory to further refine types of ENS cells that are transduced. Understanding delivery methods, AAV tropism and spread is an initial step toward the ultimate goal of therapy development. The largest obstacle for gene therapy for ENS dysfunction is identifying potential genes for repair. Few GI diseases and endophenotypes have identified gene targets, and candidate gene studies have largely been unsuccessful. To this end, AAVs are excellent tools for both research and translational purposes. As molecular biology tools, AAVs present a great opportunity for creating novel animal models of GI disease. AAVs used in combination with RNAi technology (for review see Borel et al., 2014) could be useful for identifying new gene targets and replicating symptomology that occurs in GI disease in animal models.

ENS gene therapy should be applied to diseases with identified ENS abnormalities including Hirschsprung's disease, ganglioneuromas, intestinal neuronal dysplasia, intestinal pseudo-obstruction, hypertrophic pyloric stenosis, and diabetic gastroparesis (Langer et al., 1995, Kapur, 2000, Camilleri et al., 2011, Obermayr et al., 2013). Enteric neuronal death has previously been reported in some inflammatory gastrointestinal disorders (Neunlist et al., 2003a, Villanacci et al., 2008, Venkataramana et al., 2015) and during typical aging (Camilleri et al., 2008), therefore ENS gene therapy may be used to halt neurodegeneration or for neuroregeneration. Monogenic diseases with a GI component, such as cystic fibrosis (Eggermont, 1996), will likely qualify as the first diseases that can be treated with our current AAV understanding. Furthermore, ENS gene therapy may also be important in classically categorized neurodegenerative CNS diseases that have a high comorbidity of GI symptoms. *Ex vivo* enteric neuron transfection and CNS transplantation has been suggested as a method of treating CNS damage and disease (Howard et al., 1997)

In conclusion, recent research shows that AAV gene therapy to the ENS is feasible. Gene delivery can be customized to specific GI regions and specific cell types. Improved vector design and understanding will only increase the utility of enteric gene delivery. Finally, future studies will test the efficacy of this therapeutic technique in GI disease.

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