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TECHNICAL NOTE

Proof-of-concept: neonatal intravenous injection of adeno-associated virus vectors results in successful transduction of myenteric and submucosal neurons in the mouse small and large intestine

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Key Messages

- The efficiency of transduction of both myenteric and submucosal neurons in the mouse intestine after neonatal systemic delivery of vectors AAV8 and AAV9 was investigated.
- AAV8 and AAV9 are equally capable of transducing the ENS, although the transduction efficiency in the submucosal plexus is region-dependent.
- Manipulation of the ENS through gene delivery offers great potential in preclinical research and gene therapy.

Abstract

Background *Despite the success of viral vector technology in the transduction of the central nervous system in both preclinical research and gene therapy, its potential in neurogastroenterological research remains largely unexploited. This study asked whether and to what extent myenteric and submucosal neurons in the ileum and distal colon of the mouse were transduced after neonatal systemic delivery of recombinant adeno-associated viral vectors (AAVs).* ***Methods*** *Mice were intravenously injected at postnatal day one with AAV pseudotypes AAV8 or AAV9 carrying a cassette encoding enhanced green fluorescent protein (eGFP) as a reporter under the control of a cytomegalovirus promoter. At postnatal day 35, transduction of the myenteric and submucosal plexuses of the ileum and distal colon was evaluated in whole-mount preparations, using immunohistochemistry to neurochemically identify transduced enteric neurons.* ***Key Results*** *The pseudotypes AAV8 and AAV9 showed equal potential in transducing the enteric nervous system (ENS), with 25–30% of the*

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neurons expressing eGFP. However, the percentage of eGFP-expressing colonic submucosal neurons was significantly lower. Neurochemical analysis showed that all enteric neuron subtypes, but not glia, expressed the reporter protein. Intrinsic sensory neurons were most efficiently transduced as nearly 80% of calcitonin gene-related peptide-positive neurons expressed the transgene. ***Conclusions & Inferences*** *The pseudotypes AAV8 and AAV9 can be employed for gene delivery to both the myenteric and the submucosal plexus, although the transduction efficiency in the latter is region-dependent. These findings open perspectives for novel preclinical applications aimed at manipulating and imaging the ENS in the short term, and in gene therapy in the longer term.*

Keywords *AAV, enteric nervous system, myenteric plexus, submucosal plexus, viral transfection.*

INTRODUCTION

Viral vector technology in gene delivery to the enteric nervous system (ENS) is poorly exploited, despite important merits of viral vectors in the transduction of the central nervous system in both preclinical research and gene therapy.[1–3] Recombinant adeno-associated viruses vectors (AAVs) belong to the most promising candidate vector systems in gene therapy and preclinical research[1,3], but hitherto little is known about the transduction efficiency of the ENS by AAV.

Rahim *et al.*[4] and Schuster *et al.*[5] have preliminarily indicated transduction of the myenteric plexus of the mouse with AAV9, and a more recent paper by Gombash *et al.*[6] has detailed myenteric plexus transduction by self-complementary AAV9 in neonate and juvenile mice with green fluorescent protein (GFP) being expressed under a chicken-β-actin/cytomegalovirus (CB) hybrid promoter. However, data on submucosal plexus transduction are currently lacking, as is quantification of the neuronal subtypes transduced by AAV. Here, we further explored the transduction of the ENS in the ileum and colon of the mouse by single stranded AAV8 and AAV9 encoding GFP driven by the immediately early human cytomegalovirus (CMV) promoter after neonatal i.v. injection.

MATERIALS AND METHODS

Recombinant AAV vector preparation

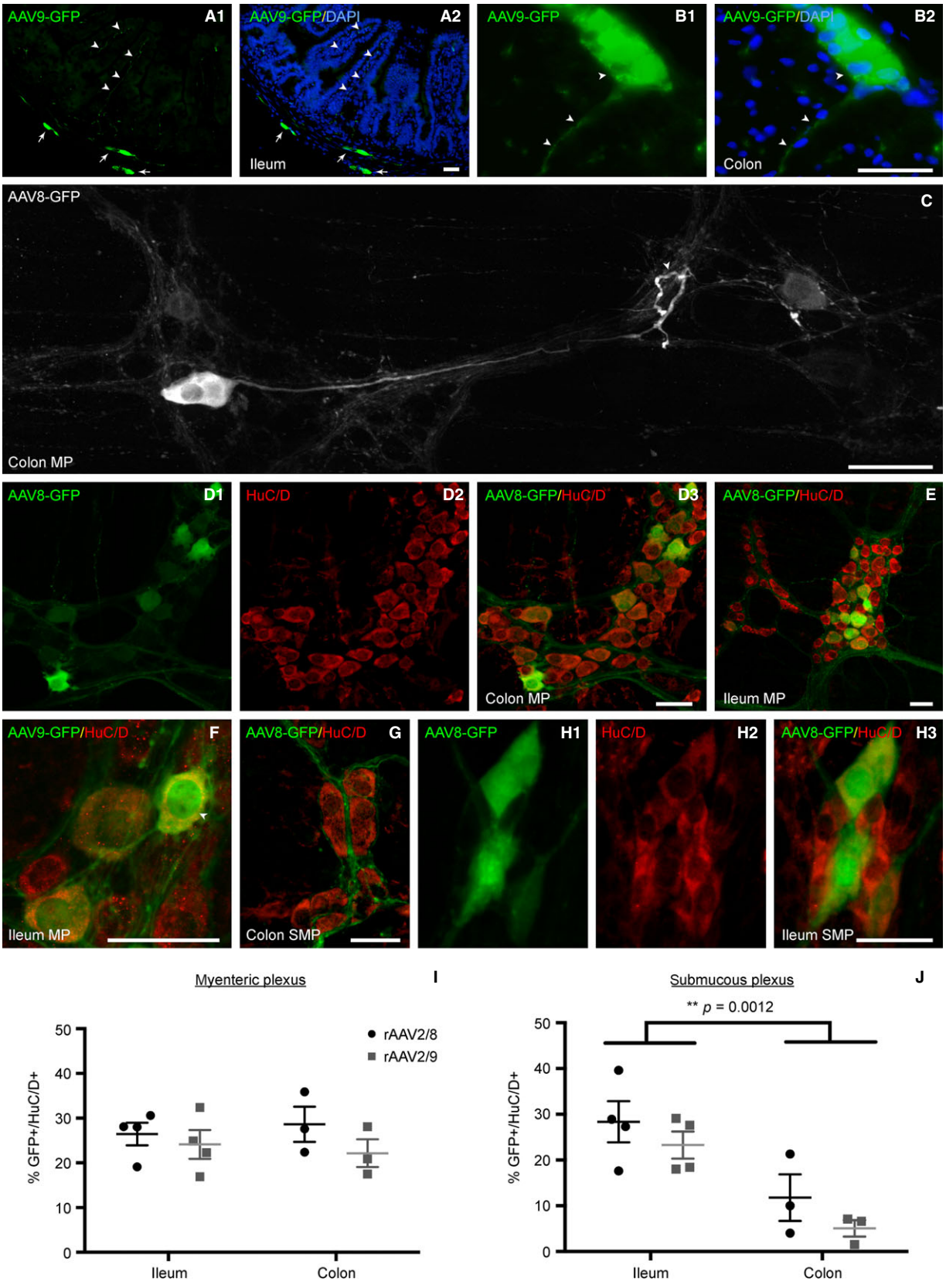
Vector production and purification were performed at the Leuven Viral Vector Core as previously described.[7] Adeno-associated viral vectors encoding enhanced GFP (eGFP) reporter protein driven by the CMV promoter were packaged in an AAV8- or AAV9-capsid. Briefly, following triple transient transfection (pAdvDeltaF6 [adenoviral helper plasmid], pAAV2/8 or pAAV2/9 [AAV serotypes], pAAV-TF CMV-eGFP-T2A-fLuc [AAV transfer plasmid encoding eGFP and fLuc reporters driven by a CMV promoter[7]] in a 1 : 1 : 1

Table 1 Total number of counted neurons in the quantification experiments

	AAV8				AAV9			
	Ileum		Distal colon		Ileum		Distal colon	
Marker	Marker+	GFP+	Marker+	GFP+	Marker+	GFP+	Marker+	GFP+
HuC/D (MP)	2950	804	2533	761	2923	704	1839	394
HuC/D (SMP)	752	226	588	65	395	96	234	11
CGRP (MP)	349	867	208	378	312	762	175	660
CB (MP)	152	563	120	300	93	472	111	599
CRT (MP)	285	351	384	441	239	234	317	319
CRT (SMP)	386	96	nd	nd	414	127	nd	nd
nNOS (MP)	332	358	739	416	472	537	898	636
VIP (SMP)	245	159	nd	nd	182	88	nd	nd

Numbers are the total of counted cells from three (neurochemical coding) or four (HuC/D stains) animals. For statistical analysis an animal was considered the experimental unit and the countings were averaged per animal. MP, myenteric plexus; SMP, submucosal plexus; AAV, adeno-associated viral vectors; GFP, green fluorescent protein; CGRP, calcitonin gene-related peptide; CRT, calretinin; VIP, vasoactive intestinal peptide.

Figure 1 Transduction of myenteric and submucosal neurons of ileum and colon. (A and B) Expression of the GFP transgene was limited to enteric ganglia (arrows) and neuronal fibers (arrowheads) (C) Transduction allows some axons to be traced along internodal strands, as exemplified by this stacked confocal image (z = 19 μm) with an axon extending into the longitudinal muscle layer (arrowhead). (D–F) Transduction of myenteric ganglia of the colon (D) and ileum (E and F). Note the lamellar dendrites on the perikaryum in (F), indicative of a Dogiel type I neuron (arrowhead). (G) GFP-expressing submucosal neurons were rare in the colon, but GFP-positive fibers could readily be observed. (H) Submucosal ganglia of the ileum showed numerous transduced neurons. (I and J) Quantification of the number of transduced neurons showed transduction efficiency to be independent of AAV serotype, but the colonic submucosal neurons were significantly less transduced than ileal submucosal neurons. Scale bars 35 μm; MP, myenteric plexus; SMP, submucosal plexus; AAV, adeno-associated virus.



ratio) into HEK293T cells using 25 kDa linear polyethylenimine, viral vector particles were collected from the supernatant and concentrated using tangential flow filtration and iodixanol gradient purification. Gradient fractions of concentrated AAV particles were stored at −80 °C. Titers for AAV stocks were controlled by qPCR analysis to determine AAV genome copies (GC/mL) and silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Injection procedure

Following hypothermic anesthesia, the respective AAV vectors were administered to C57BL/6 mouse pups on postnatal day one by injection into the superficial temporal vein using a 33G needle (Hamilton, Reno, NV, USA). 20 μL of AAV vector with 1.09×10^{12} GC/mL AAV8 or 1.19×10^{12} GC/mL AAV9 was administered. Eight animals from two different litters were injected for each vector. At 35 days of age the injected mice were sacrificed. All animal experiments were approved by the Ethical Committee for Animals of the University of Antwerp and were in line with EU directive 2010/63/EU.

Immunohistochemistry

The ileum and distal colon were fixed in 4% paraformaldehyde. Whole-mounts and cryosections were prepared as described before.[8,9] Tissue samples were preincubated in 0.1 M phosphate-buffered saline (PBS; pH 7.4) with 0.05% thimerosal, 10% normal horse serum, and 1% triton X-100. In case of anti-HuC/D staining this was supplemented with Mouse-On-Mouse blocking reagent (Vector Labs, Burlingame, CA, USA). Tissue was incubated overnight with one of the following primary antibodies; mouse anti-HuC/D (1 : 500; Thermo Fisher Scientific, Waltham, MA, USA; clone 16A11), goat anti-neuronal nitric oxide synthase (nNOS; 1 : 1000; Abcam, Cambridge, MA, USA; ab1376), goat anti-calbindin (CALB; 1 : 200; Santa Cruz Biotechnology, Dallas, TX, USA; sc-7691), goat anti-calretinin (CRT; 1 : 10 000; Swant, Marly, Switzerland; CG1), sheep anti-tyrosine hydroxylase (TH; 1 : 500; Novus Biologicals, Littleton, CO, USA; NB300-110), goat anti-vasoactive intestinal peptide (VIP; 1 : 100; Santa Cruz Biotechnology sc-7841), rabbit anti-S100B (1 : 5000; Dako, Glostrup, Denmark; Z0311), goat anti-calcitonin gene-related peptide (CGRP; 1 : 5000; Abcam ab36001), rabbit anti-glial fibrillary acidic protein (GFAP; 1 : 500; Dako Z0334), or rabbit anti-GFP (1 : 500; Abcam ab290). After rinsing with PBS, tissues were incubated with corresponding Cy3-conjugated secondary IgG antibodies raised in donkey (1 : 800; Jackson Immunoresearch, West Grove, PA, USA). Antibodies were validated by appropriate negative control experiments as detailed previously.[10,11] In case of VIP and CGRP immunostaining for quantification, axonal transport was blocked with colchicine in organotypic culture enhancing the perikaryal localization of these neuropeptides. The culture medium was composed of 10% FCS, 100 U/mL penicillin–streptomycin, 50 μg/mL gentamycin, 2.5 μg/mL amphotericin B, 1 μM nifedipine, 0.1 mg/mL colchicine in DMEM:F12 (Life Technologies). Images were acquired using a Zeiss (Oberkochen, Germany) Axiophot fluorescence microscope equipped with an Olympus (Tokyo, Japan) DP70 digital camera (cryosections) or a Zeiss LSM510 confocal microscope (whole-mounts).

Quantification & statistical analysis

Whole-mounts from at least three animals were counted (exact n is indicated in the results section). Randomly chosen fields of view amounted to an imaged area of 1.5 mm^2 per whole-mount. The number of counted neurons is listed in Table 1. Images were manually counted using ImageJ and data were further analyzed with Graphpad (La Jolla, CA, USA) Prism 6. Data are represented as mean ± SEM. The influence of the factors 'intestinal region' (ileum or colon) and 'AAV serotype' (AAV8 or AAV9) on the number of transduced (GFP-positive) enteric neurons (HuC/D-positive) was statistically evaluated with two-way ANOVA at a $p = 0.05$ significance level.

RESULTS

Transduction of the myenteric and submucosal plexuses by AAV8 and AAV9

Green fluorescent protein fluorescence was limited to enteric ganglia and interganglionic connecting nerve strands, with an identical GFP pattern for AAV8 or AAV9 (Fig. 1A and B). Endogenous GFP fluorescence coincided with anti-GFP immunoreactivity in the whole-mounts, indicating that endogenous GFP fluorescence had not suffered from fixation or staining procedures (data not shown). Adeno-associated viral vector-transduced GFP fluorescence allowed morphological classification into Dogiel subtypes and tracing of individual axons along internodal strands in whole-mount preparations (Fig. 1C). The pseudotypes, AAV8 and AAV9, were equally potent in transducing the ileal and colonic enteric plexuses (Table 2).

The myenteric plexus of both regions was transduced to the same extent, with 25–30% of the HuC/D immunoreactive myenteric neurons coexpressing GFP (Fig. 1D–F, Table 2). However, a significantly lower number of submucosal neurons showed GFP fluorescence in the colon ($p = 0.0012$, two-way ANOVA), although GFP-fluorescent nerve fibers could be readily observed (Fig. 1G and H, Table 2).

Transduction of the neurochemical classes of enteric neurons by AAV8 and AAV9

All neuronal subtypes were susceptible to AAV transduction, as revealed by neurochemical marker staining (Fig. 2, Table 3). Given the low transduction of the colonic submucosal plexus, subtypes were not quantified for this specific region. The highest transduction efficiency was observed in the CGRP- or CALB-immunoreactive myenteric neurons, with 50 to almost 80% of these neurons expressing GFP. In the other subpopulations the transduced proportion remained closer to the 20–30% range. About 50 ganglia from two animals per AAV serotype were evaluated for the glial cell markers GFAP or S100. None of them yielded any GFP signal in enteric glial cells (Fig. 2I and J).

Table 2 Transduction of enteric neurons by AAV8 or AAV9

GFP+/HuC-D+, mean % ± SEM (n = 4 animals)	AAV8		AAV9	
	Ileum	Colon	Ileum	Colon
Myenteric plexus	26 ± 3	29 ± 4	24 ± 3	22 ± 3
Submucosal plexus	28 ± 5	12 ± 5	23 ± 3	5 ± 2

See Table 1 for abbreviations.

DISCUSSION

We demonstrate that AAV8 and AAV9, carrying GFP under control of a CMV promoter, efficiently transduce myenteric and submucosal neurons of the mouse small and large intestine when injected i.v. in P1 neonates. This is in line with Gombash *et al.*, who recently employed AAV vectors containing a self-complementary

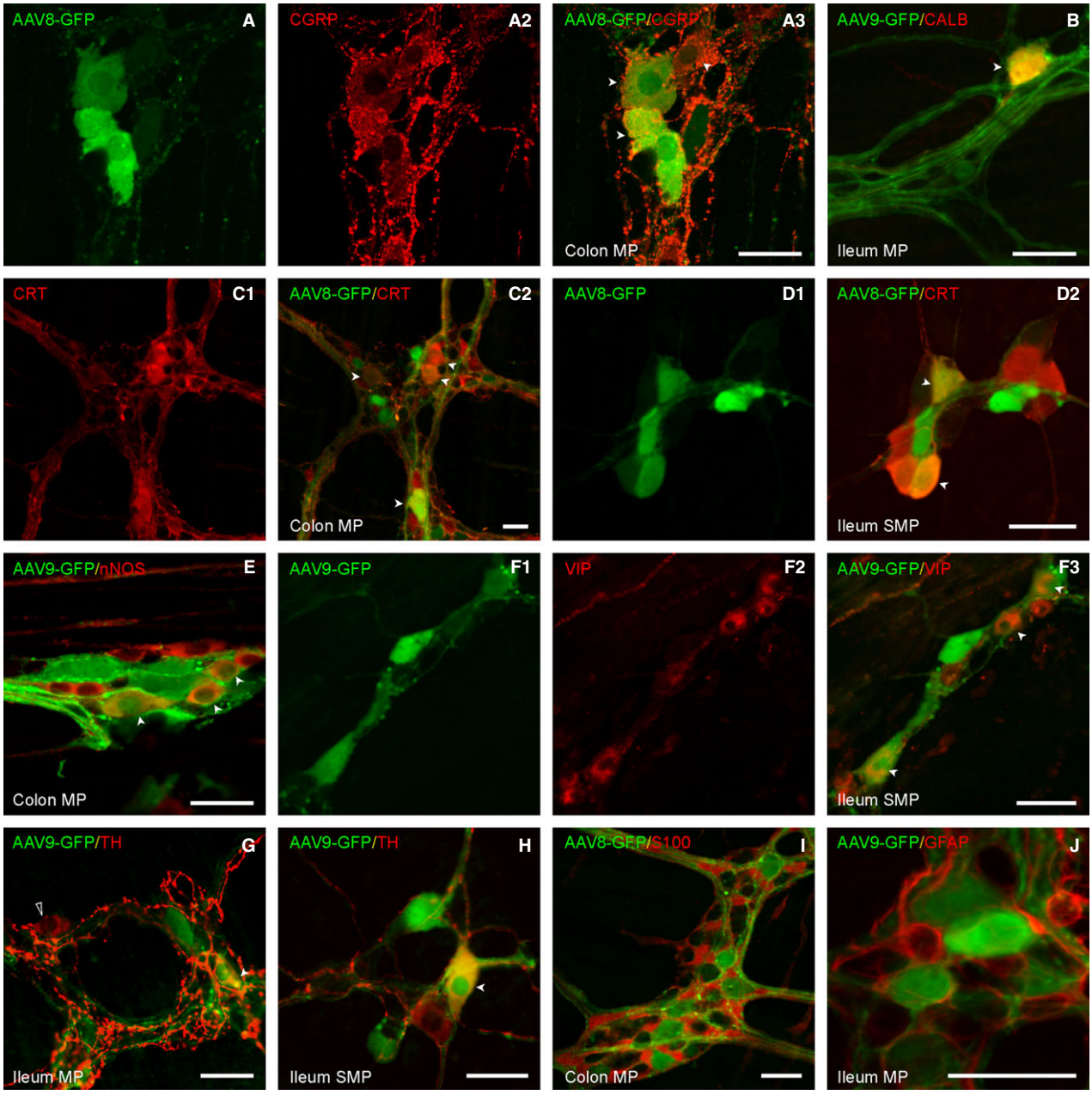


Figure 2 Neurochemical classification of transduced myenteric and submucosal neurons. Neurons immunostained for (A) calcitonin gene-related peptide, (B) calbindin, (C and D) calretinin, (E) neuronal nitric oxide synthase, (D) vasoactive intestinal peptide, or (F and G) tyrosine hydroxylase all were susceptible to transduction with AAV8 and AAV9. (H and I) S100 (H) or glial fibrillary acidic protein (I) immunoreactive glial cells did not express the transgene. Scale bars 35 μm; MP, myenteric plexus; SMP, submucosal plexus; AAV, adeno-associated virus.

Table 3 Transduction in neurochemically stained enteric neurons

	AAV8				AAV9			
	Ileum		Distal colon		Ileum		Distal colon	
Mean % ± SEM (n = 3)	GFP+/ marker+	Marker+/ GFP+	GFP+/ marker+	Marker+/ GFP+	GFP+/ marker+	Marker+/ GFP+	GFP+/ marker+	Marker+/ GFP+
CGRP (MP)	78 ± 1	31 ± 2	60 ± 2	31 ± 1	77 ± 5	32 ± 4	57 ± 11	18 ± 4
CB (MP)	54 ± 6	16 ± 2	49 ± 6	19 ± 0	61 ± 5	11 ± 2	70 ± 2	10 ± 1
CRT (MP)	33 ± 5	27 ± 3	37 ± 2	31 ± 3	27 ± 1	26 ± 3	40 ± 18	30 ± 6
CRT (SMP)	14 ± 4	50 ± 12	nd	nd	18 ± 1	54 ± 1	nd	nd
nNOS (MP)	17 ± 2	16 ± 1	15 ± 2	31 ± 5	21 ± 7	17 ± 2	16 ± 4	22 ± 3
VIP (SMP)	32 ± 5	45 ± 7	nd	nd	52 ± 5	73 ± 3	nd	nd

In the myenteric plexus CGRP is expressed in intrinsic primary afferent neurons (IPANs); CB marks a subpopulation of IPANs; nNOS is mainly found in inhibitory motor neurons; CRT marks IPANs, excitatory motor neurons, and a subpopulation of interneurons. In the submucosal plexus CRT and VIP stain vasodilator and secretomotor neurons. A detailed description of the different neurochemical classes in the mouse enteric nervous system can be found in references (17–19). See Table 1 for abbreviations.

genome encoding GFP under a CB promoter for the transduction of the myenteric plexus.[6] Hence, the rate-limiting second-strand synthesis required in the case of single stranded AAV, does not affect the construct's expression in enteric neurons. The advantage of using single stranded AAV vectors lies in the longer construct length (4.7 kb) they can hold compared to self-complementary AAV (about 2.4 kb). Both vectors were equally potent, in contrast to the lower transduction efficiency of AAV8 compared to AAV9 observed in the adult rat colon after intramural injection of AAV-GFP under the CB promoter.[12] It should be noted that the systemic distribution of intravenously injected AAV is not limited to the ENS.[4,5,13] In the light of gene therapy, future efforts should evaluate specificity-enhancing strategies such as ENS-specific promotors, AAV with modified glycan binding ability or micro-RNAs.[14,15] Enteric glia lacked transgene expression, but these cells could be targeted with other AAV serotypes or GFAP promotor-driven constructs, as these strategies have been proven successful in earlier work.[6,12]

To our knowledge, the paper by Gombash *et al.* is the only other work that neurochemically identifies AAV-transduced myenteric neurons of the mouse.[6] Our results further revealed significantly lower transduction efficiency in the submucosal plexus of the distal colon, compared to the ileum. The reason for this difference is not clear: transduction efficiency can be affected due to anatomical constraints such as villous fenestrated capillaries in the ileum facilitating viral vector access, or pertain to more complex physiological differences such as regional differences in immune response.[16] Alternatively, even though the CMV promoter can be considered as an ubiquitous promoter, lower CMV-driven expression in the submucosal plexus of the distal colon may also account for this difference.

Gombash *et al.* did not quantify transduction in neurochemically coded neurons, but did report that GFP expression was absent in VIP- or nNOS-positive myenteric neurons after AAV9 transduction, which contrasts with our observations showing nearly 20% of inhibitory (nitrergic) motor neurons expressing GFP.[6] The proportion of transduced intrinsic sensory neurons (CGRP- or CALB-immunoreactive) was substantially larger compared to inhibitory motor neurons, but transduction of the latter was not absent. Moreover, submucosal VIPergic neurons were readily transduced in our study. These discrepant observations might pertain to differences in the promoter (CB *vs* CMV), although the genetic background of the mice (FVB *vs* C57BL/6) may also play a role.

This study strengthens the validity of AAV vectors in transducing the myenteric plexus of the mouse and expands AAV application to the submucosal plexus. This application has important preclinical merits on the short term: Cre-inducible transgene-cassettes combined with Cre-expressing mouse lines enables selective manipulation of enteric neurons *in vivo*. For example, transduction of genetically encoded calcium sensors or channel rhodopsins allows selective imaging and manipulation in live cell experiments. The fact that transduction can be executed in the neonatal time window is important for studying postnatal ENS development during the weaning period. In the longer term, genetic therapies targeting the ENS in clinical applications can be evaluated.

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DISCLOSURE

No competing interest declared.

AUTHOR CONTRIBUTION

RB designed the study, obtained funding, performed experiments, analyzed data, and wrote the paper; SVR performed experiments; RG contributed the AAV vectors; SW participated in study design and provided technical and scientific support for the study; JPT designed the study, obtained funding, and wrote the paper. All authors critically reviewed the manuscript.

REFERENCES

1 Lentz TB, Gray SJ, Samulski RJ. Viral vectors for gene delivery to the central nervous system. *Neurobiol Dis* 2012; **48**: 179–88.
2 Murlidharan G, Samulski RJ, Asokan A. Biology of adeno-associated viral vectors in the central nervous system. *Front Mol Neurosci* 2014; **7**: 1–9.
3 McCown TJ. Adeno-Associated Virus (AAV) Vectors in the CNS. *Curr Gene Ther* 2011; **11**: 181–8.
4 Rahim A, Wong AMS, Hoefer K, Buckley SMK, Mattar CN, Cheng SH, Chan JKY, Cooper JD *et al.* Intravenous administration of AAV2/9 to the fetal and neonatal mouse leads to differential targeting of CNS cell types and extensive transduction of the nervous system. *FASEB J* 2011; **25**: 3505–18.
5 Schuster DJ, Dykstra JA, Riedl MS, Kitto KF, Belur LR, McIvor RS, Elde RP, Fairbanks CA *et al.* Biodistribution of adeno-associated virus serotype 9 (AAV9) vector after intrathecal and intravenous delivery in mouse. *Front Neuroanat* 2014; **8**: 42.
6 Gombash SE, Cowley CJ, Fitzgerald JA, Hall JCE, Mueller C, Christofi FL, Foust KD. Intravenous AAV9 efficiently transduces myenteric neurons in neonate and juvenile mice. *Front Mol Neurosci* 2014; **7**: 81.
7 Van der Perren A, Toelen J, Carlon M, Van den Haute C, Coun F, Heeman B, Reumers V, Vandenberghe LH *et al.* Efficient and stable transduction of dopaminergic neurons in rat substantia nigra by rAAV 2/1, 2/2, 2/5, 2/6.2, 2/7, 2/8 and 2/9. *Gene Ther* 2011; **18**: 517–27.
8 Buckinx R, Bagyanszki M, Avula LR, Adriaensen D, Van Nassauw L, Timmermans J-P. Expression of corticotropin-releasing factor and urocortins in the normal and Schistosoma mansoni-infected mouse ileum. *Cell Tissue Res* 2014; **359**: 453–63.
9 Avula LR, Buckinx R, Alpaerts K, Costagliola A, Adriaensen D, Van Nassauw L, Timmermans J-P. The effect of inflammation on the expression and distribution of the MAS-related gene receptors MrgE and MrgF in the murine ileum. *Histochem Cell Biol* 2011; **136**: 569–85.
10 Van Op den Bosch J, van Nassauw L, Lantermann K, van Marck E, Timmermans J-P. Effect of intestinal inflammation on the cell-specific expression of somatostatin receptor subtypes in the murine ileum. *Neurogastroenterol Motil* 2007; **19**: 596–606.
11 Buckinx R, Bagyanszki M, Avula LR, Adriaensen D, Van Nassauw L, Timmermans J-P. Expression of corticotropin-releasing factor and urocortins in the normal and Schistosoma mansoni-infected mouse ileum. *Cell Tissue Res* 2015; **359**: 453–63.
12 Benskey MJ, Kuhn NC, Galligan JJ, Garcia J, Boye SE, Hauswirth WW, Mueller C, Boye SL *et al.* Targeted gene delivery to the enteric nervous system using AAV: a comparison across serotypes and capsid mutants. *Mol Ther* 2015; **23**: 488–500.
13 Mattar CN, Wong AMS, Hoefer K, Alonso-Ferrero ME, Buckley SMK, Howe SJ, Cooper JD, Waddington SN *et al.* Systemic gene delivery following intravenous administration of AAV9 to fetal and neonatal mice and late-gestation nonhuman primates. *FASEB J* 2015; **29**: 3876–88.
14 Shen S, Bryant KD, Sun J, Brown SM, Troupes A, Pulicherla N, Asokan A. Glycan binding avidity determines the systemic fate of adeno-associated virus type 9. *J Virol* 2012; **86**: 10408–17.
15 Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A, Baccarini A, Lazzari G *et al.* Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol* 2007; **25**: 1457–67.
16 Mowat AM, Agace WW. Regional specialization within the intestinal immune system. *Nat Rev Immunol* 2014; **14**: 667–85.
17 Sang Q, Young HM. Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. *Cell Tissue Res* 1996; **284**: 39–53.
18 Qu Z-D, Thacker M, Castelucci P, Bagyánszki M, Epstein ML, Furness JB. Immunohistochemical analysis of neuron types in the mouse small intestine. *Cell Tissue Res* 2008; **334**: 147–61.
19 Mongardi Fantaguzzi C, Thacker M, Chiocchetti R, Furness JB. Identification of neuron types in the submucosal ganglia of the mouse ileum. *Cell Tissue Res* 2009; **336**: 179–89.