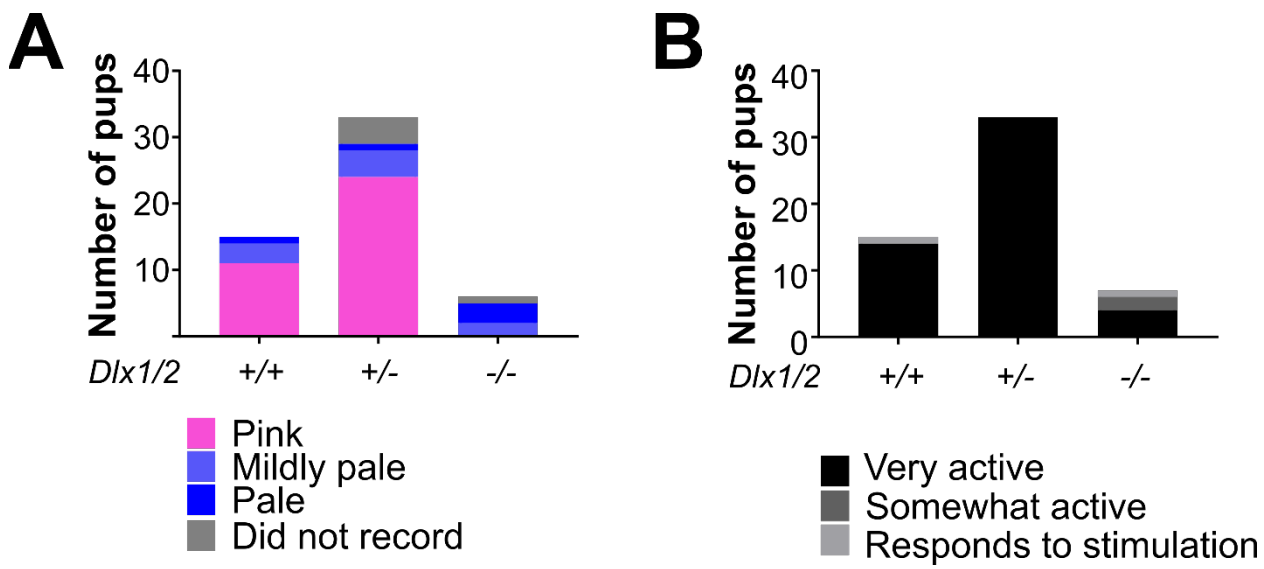
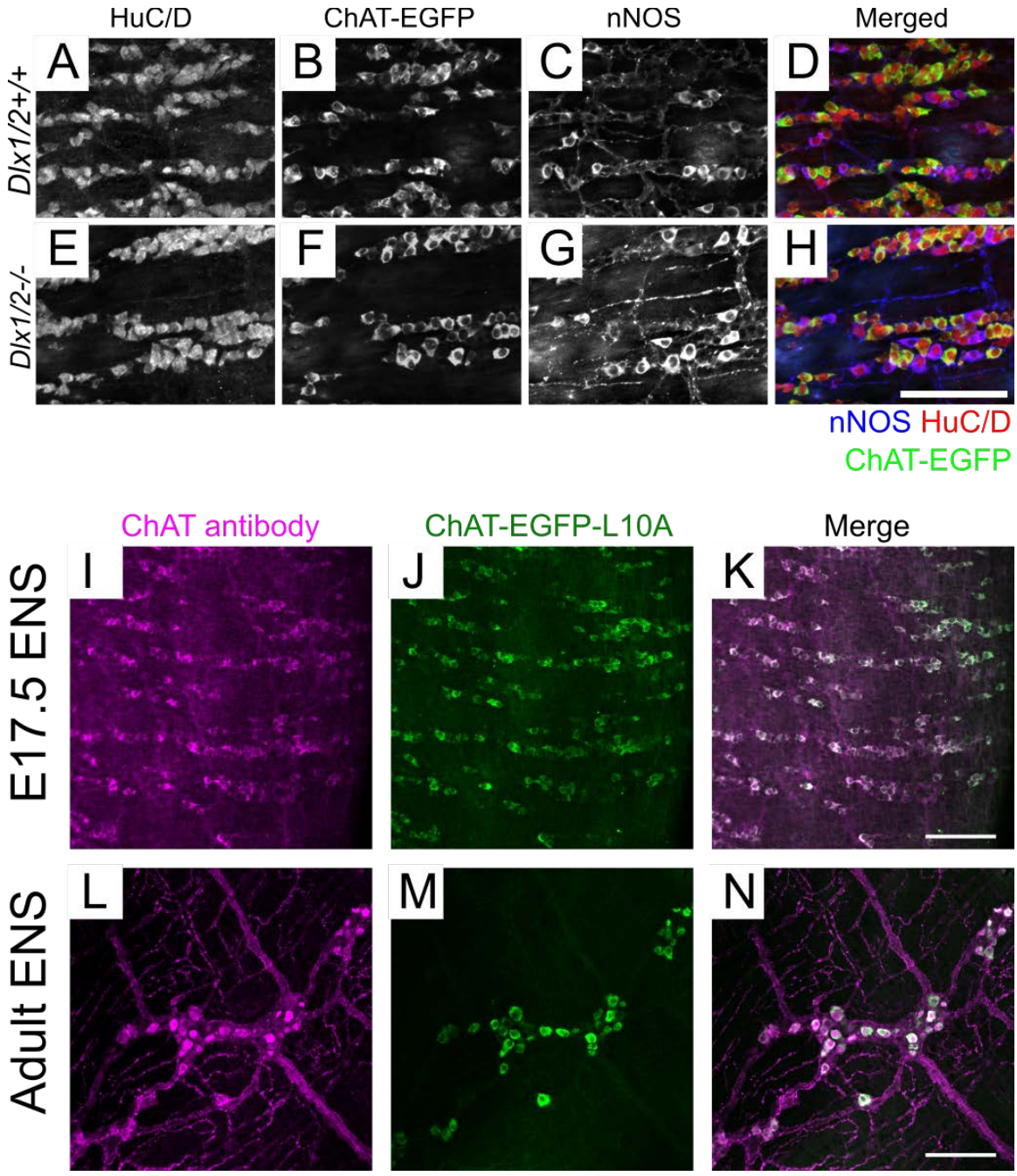
Supplementary material:



Supplementary Figure 1:

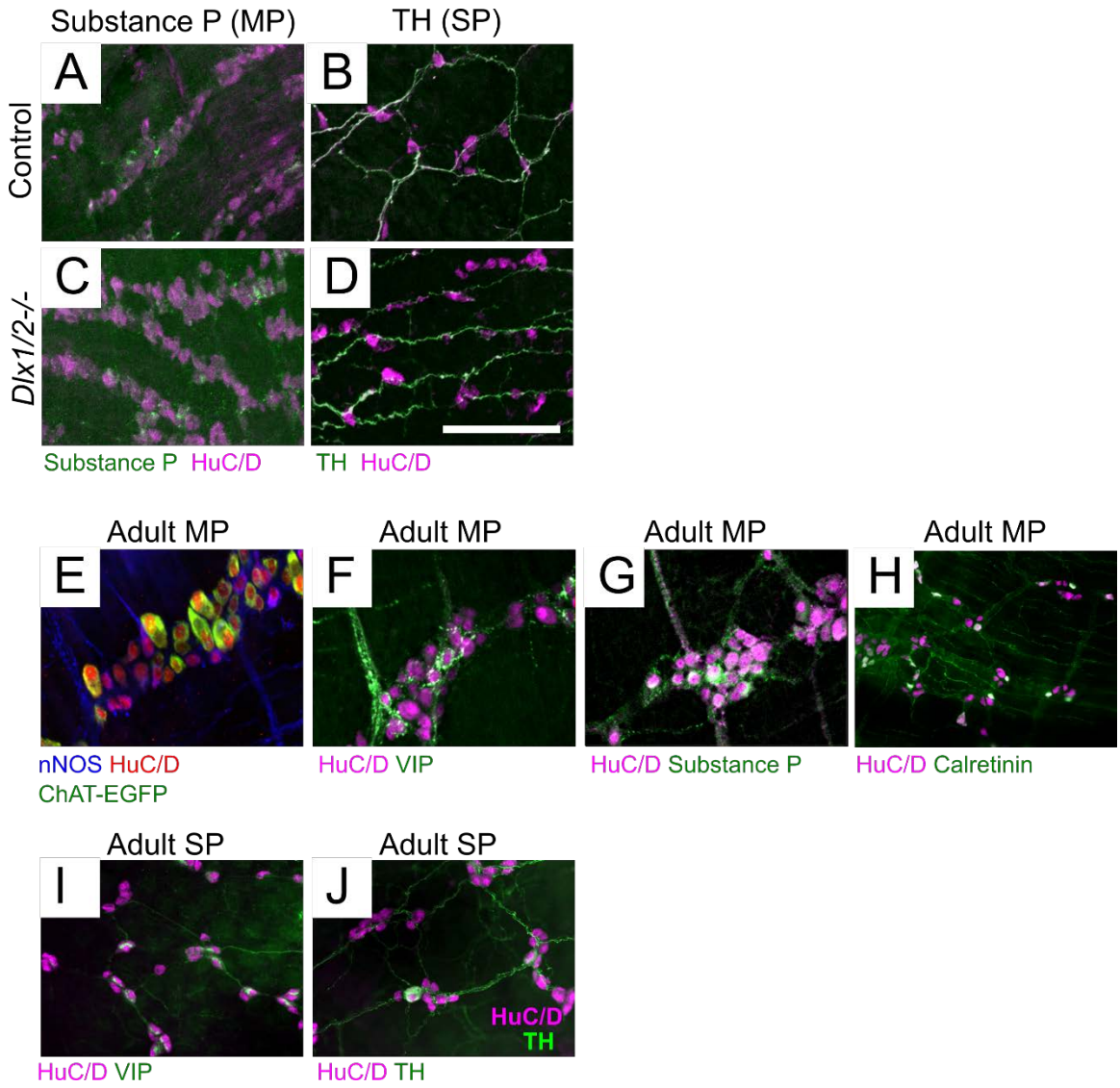
(A-B) A higher proportion of *Dlx1/2-/-* mice given FITC dextran were pale-appearing (A) and exhibited fewer spontaneous movements (B) compared to wild-type and heterozygous controls.



Supplementary Figure 2:

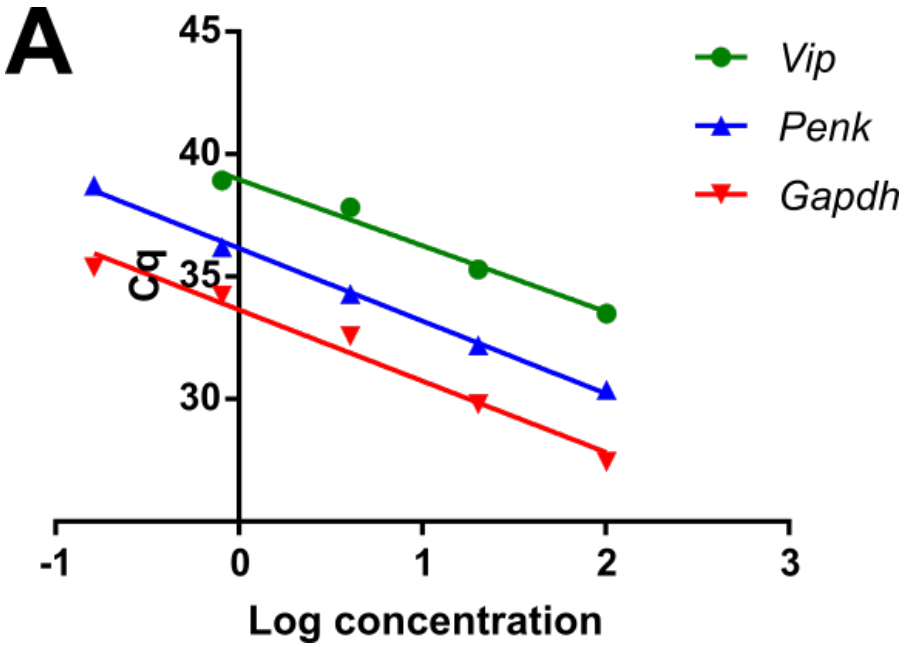
(A-H) Colorblind-friendly separation of channels for *Dlx1/2;ChAT-EGFP* images shown in Figure 6A (A-D) and Figure 6G (E-H). (I-N) Immunostaining small intestine with a

ChAT antibody confirms colocalization with EGFP at E17.5 (I-K) and in adulthood (L-N).



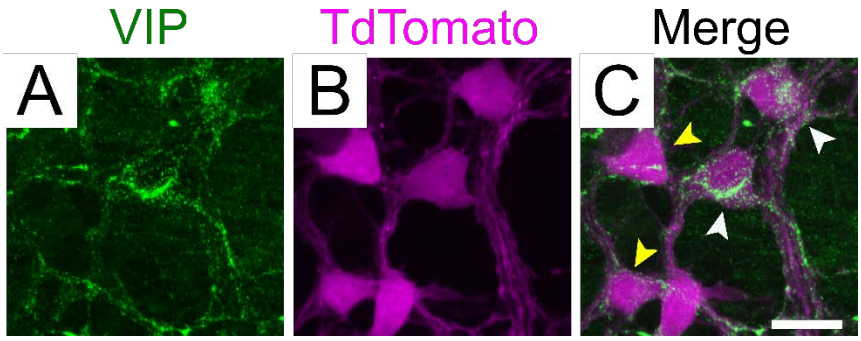
Supplementary Figure 3:

(A-D) Myenteric plexus (MP) substance P (A,C) and submucosal plexus (SP) tyrosine hydroxylase (TH; B,D) appeared normal in *Dlx1/2-/-* P0 small intestine, but we did not perform quantifications. (E-J) Positive control immunostaining in adult mouse small intestine for antibodies used in Figure 6. Scale bar = 100 μm.



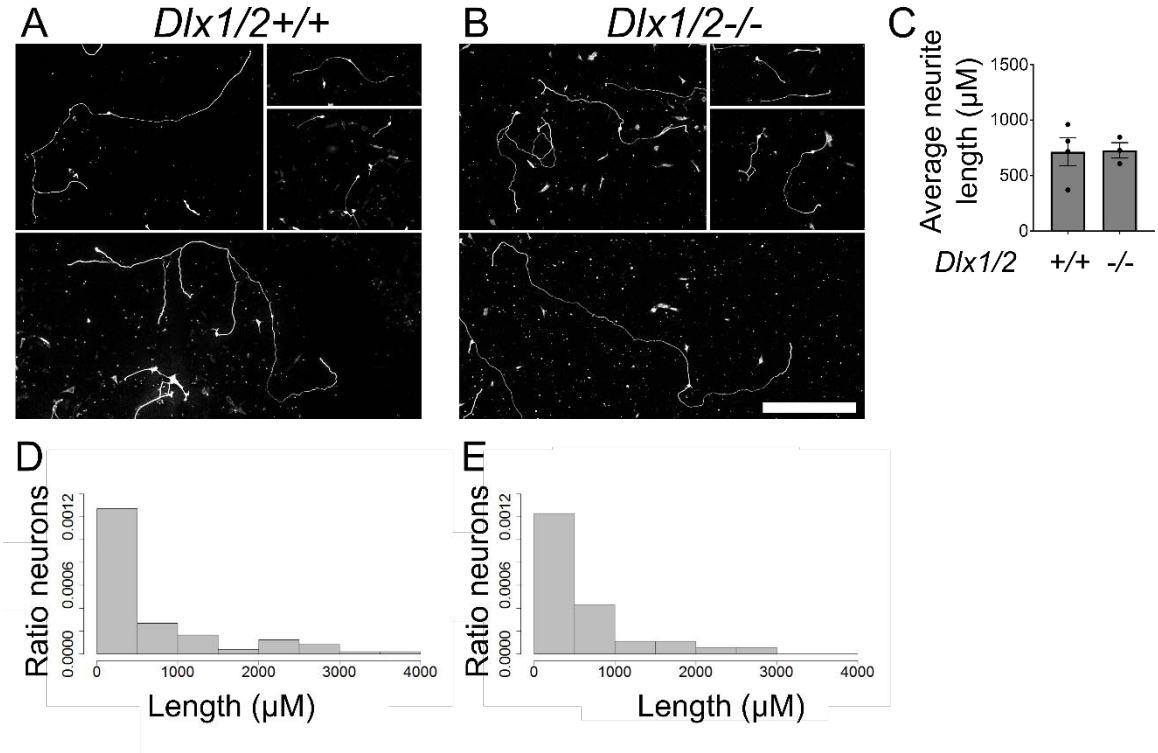
Supplementary Figure 4:

(A) qPCR standard curve for validation of *Vip*, *Penk*, and *Gapdh*.



Supplementary Figure 5:

(A-C) VIP (green, A) and TdTomato (magenta, B) co-localization in a *Dlx1/2;Vip-Ires-Cre;TdTomato* control P0 mouse. White arrowheads indicate high VIP expressing cells; yellow arrowheads indicate low VIP expressors. Scale bar is 20 microns.



Supplementary Figure 6: Cultured *Dlx1/2-/-* enteric neurons show no neurite length differences in vitro. (A-B) Representative images of cultured WT (A) and *Dlx1/2-/-* (B) neurons stained with TuJ1 antibody. (C) Quantification of total neurite length/total neuron number (Student's t-test, N > 400 nerve cell bodies, n = 4 embryos (+/+) and n=3 embryos (-/-)). (D-E) Histograms of total neurite length in single cells where neurites could be traced unambiguously (N = 96 (*Dlx1/2+/+*), N = 70 (*Dlx1/2-/-*)). Scale bar = 500 μm and applies to all images shown.

Supplementary Table 1: Genotyping primers

Primer name	**Sequence**	**Genotyping solution**
Dlx1/2 and *Dlx2* mutant F	5′ CTA CTC CGC CAA AAG CAG CTA CGA CC 3′	KAPA (Roche)
Dlx1/2 and *Dlx2* mutant R	5′ GCC AGC TCA TTC CTC CCA CTC ATG ATC 3′	KAPA
Dlx1/2 and *Dlx2* wild type F	5′ GCT GAT GGA TGA GCT CTA AGT ATG 3′	KAPA
Dlx1/2 and *Dlx2* wild type R	5′ ACG CAC CAT CTA CTC CAG TTT 3′	KAPA
Dlx1 wild type F	5′ AAC CCC TGT TCC GCT TAA ATT GGG TTC CTT C 3′	KAPA
Dlx1 wild type R	5′ GTG GCT GCT GAC CGA GTT GAC GTA GG 3′	KAPA
Cre wild type F	5′ GCA TTA CCG GTC GAT GCA ACG AGT GAT GAG 3′	KAPA
Cre wild type R	5′ GAG TGA ACG AAC CTG GTC GAA ATC AGT GCG 3′	KAPA
GFP transgene F	5′ GCA CGA CTT CTT CAA GTC CGC CAT GCC 3′	KAPA

GFP transgene R	5′ GCG GAT CTT GAA GTT CAC CTT GAT GCC 3′	KAPA
Tdtomato mutant F	5′ ACT ACT ACG TGG ACA CCA AGC TGG ACA TCA 3′	KAPA
Tdtomato mutant R	5′ GGC ATT AAA GCA GCG TAT CCA CAT AGC GTA 3′	KAPA
Tdtomato WT F	5′ GTT ATC AGT AAG GGA GCT GCA GTG GAG TAG 3′	GoTaq Green Master Mix (Promega, Madison, WI)
Tdtomato WT R	5′ CCG AAA ATC TGT GGG AAG TCT TGT CCC TCC 3′	GoTaq Green Master Mix

53 **Supplementary Table 2: List of antibodies**

Antibody	**Concentration**	**Catalog number**	**Source**
Rabbit anti-TuJ1	1:10,000	PRB-435P	Covance (Princeton, NJ); RRID:AB_10063850
Rabbit anti-nNOS	1:200	AB5380	Chemicon/Millipore (Burlington, MA); RRID:AB_91824
Rabbit anti-GABA	1:200	A2052	Sigma; RRID:AB_477652
Rat anti-Somatostatin	1:500	MAB354	Millipore; RRID:AB_2255365
Rabbit anti-Calretinin	1:5000	AB5054	Chemicon; RRID:AB_2068506
Rabbit anti-vasoactive intestinal peptide	1:300	20077	Immunostar (Hudson, WI); RRID:AB_572270
Sheep anti-tyrosine hydroxylase	1:500	AB152	Chemicon
ANNA-1 (HuC/D)	N/A*	N/A	Kind gift from Dr. V. Lennon, Mayo Clinic
Rabbit anti-S100β	1:200	Ab52642	Abcam; RRID:AB_882426

Goat anti-Sox10	1:200	sc-17342	Santa Cruz (Dallas, TX); RRID:AB_2195374
Goat anti-ChAT	1:100	AB144P	Millipore; RRID:AB_2079751
Chicken anti-GFP	1:200	GFP-1020	Aves Labs; RRID:AB_10000240
Alexa Fluor goat anti-human 647	1:400	A21445	Life Technologies; RRID:AB_2535862
AlexaFluor donkey anti-rabbit 488	1:400	A21206	Life Technologies; RRID:AB_2535792
AlexaFluor donkey anti-rabbit 594	1:400	A21207	Life Technologies; RRID:AB_141637
AlexaFluor donkey anti-rabbit 647	1:400	A31573	Life Technologies; RRID:AB_2536183
AlexaFluor donkey anti-rat 488	1:400	A21208	Life Technologies; RRID:AB_141709
AlexaFluor donkey anti-goat 594	1:400	A11058	Life Technologies; RRID:AB_2534105
AlexaFluor donkey anti-sheep 594	1:400	A11016	Life Technologies; RRID:AB_10562537

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* ANNA-1 is a human antibody that we use repeatedly. The concentration of primary antibody declines with each use, so we do not know the actual antibody concentration when this antibody is used on each specimen.

Supplementary Table 3: qPCR primers

Primer name	**Sequence**	**Reference**
Vip Forward	5′ GCATGCTGATGGAGTTTTCA 3′	(1)
Vip Reverse	5′ GGCATCAGAGTGTCGTTTGA 3′	(1)
Penk Forward	5’ TTCAGCAGATCGGAGGAGTTG 3’	(2)
Penk Reverse	5’ AGAAGCGAACGGAGGAGAGAT 3’	(2)
Gapdh Forward	5’ AACTTTGGCATTGTGGAAGG 3’	(3)
Gapdh Reverse	5’ GTCTTCTGGGTGGCAGTGAT 3’	(3)

Supplementary video 1:

Video of *Dlx1/2-/-* (top) and control (bottom) small intestine at baseline, at 8x speed. Bowel orientation is proximal (right) and distal (left) for all videos. In control bowel (bottom), a neurally-mediated contraction complex involving pronounced bowel straightening and complex propulsive patterns of motility up and down the bowel occurs from 00:04-00:07. At other times, the control bowel is in a baseline contracted state. In contrast, the mutant *Dlx1/2-/-* bowel is constantly in a baseline contracted state and does not straighten throughout the video.

Supplementary video 2:

Video of *Dlx1/2-/-* (top) and control (bottom) bowel after TTX treatment at 8x speed. Here, the bowels appear even more contracted and do not undergo neurally-mediated straightening.

References

1. Ngan ES, Shum CK, Poon HC, Sham MH, Garcia-Barcelo MM, Lui VC, and Tam PK. Prokineticin-1 (Prok-1) works coordinately with glial cell line-derived neurotrophic factor (GDNF) to mediate proliferation and differentiation of enteric neural crest cells. *Biochimica et biophysica acta.* 2008;1783(3):467-78.
2. Denning GM, Ackermann LW, Barna TJ, Armstrong JG, Stoll LL, Weintraub NL, and Dickson EW. Proenkephalin expression and enkephalin release are widely observed in non-neuronal tissues. *Peptides.* 2008;29(1):83-92.
3. Lopez SH, Avetisyan M, Wright CM, Mesbah K, Kelly RG, Moon AM, and Heuckeroth RO. Loss of Tbx3 in murine neural crest reduces enteric glia and causes cleft palate, but does not influence heart development or bowel transit. *Developmental biology.* 2018.