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Title: Dlx1/2 mice have abnormal enteric nervous system function

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Abstract

Decades ago, investigators reported that mice lacking DLX1 and DLX2, transcription factors expressed in the enteric nervous system (ENS), die with possible bowel motility problems. These problems were never fully elucidated. We found that mice lacking DLX1 and DLX2 (Dlx1/2-/- mice) had slower small bowel transit and reduced or absent neurally-mediated contraction complexes. In contrast, small bowel motility seemed normal in adult mice lacking DLX1 (Dlx1-/-). Even with detailed anatomic studies, we found no defects in ENS precursor migration, or neuron and glia density in Dlx1/2-/- or Dlx1-/- mice. However, RNA sequencing of Dlx1/2-/- ENS revealed dysregulation of many genes, including vasoactive intestinal peptide (Vip). Our study reveals a novel connection between Dlx genes and Vip and highlights the observation that dangerous bowel motility problems can occur in the absence of easily-identifiable ENS structural defects. These findings may be relevant for disorders like chronic intestinal pseudo-obstruction (CIPO) syndrome.
Introduction

Chronic intestinal pseudo-obstruction (CIPO) is a serious digestive disorder characterized by profound bowel motility defects, leading to severe constipation, abdominal distention, and life-threatening malnutrition requiring total parenteral nutrition (TPN). Although rare, CIPO is a leading cause of intestinal failure (1), accounting for ~10-14% of small bowel transplants (2). Many CIPO cases are believed to be caused by defects in the enteric nervous system (ENS), a complex network of neurons and glia within the bowel wall (3). The ENS develops from enteric neural crest-derived cells (ENCDC) that migrate through the bowel during fetal development (4). ENCDC give rise to diverse neuron and glia subtypes that control nutrient absorption, epithelial secretion, intestinal mixing, and (critically) transport of contents down the bowel (5). In neuropathic CIPO, neurons and glia are present, but damaged or dysfunctional, leading to dysmotility.

Neuron dysfunction in CIPO has many etiologies, including infection, autoimmune disease, neurodegeneration, and gene mutations (1, 6). Genetic causes of CIPO are poorly understood. Although genes like SOX10 and POLG have been linked to neuropathic CIPO (7, 8), we believe many CIPO-linked genes remain to be discovered (9). Here, we examine the role of Dlx1 and Dlx2, two transcription factors expressed in developing ENS. We discovered that loss of these genes in mice causes profound intestinal dysmotility despite nearly normal ENS anatomy.

Dlx1 and Dlx2 are highly conserved homeobox transcription factors located in a tail-to-tail configuration on mouse chromosome 2. They are essential for craniofacial, palate (10), tooth (11), and central nervous system (CNS) morphogenesis (10, 12-14). In CNS, Dlx1 and Dlx2 are critical for subpallial interneuron differentiation and migration.
into the cortex and olfactory bulb (13). Dlx1-/− and Dlx2-/− mice exhibit milder defects than Dlx1/2 double mutants, implying functional redundancy for DLX1 and DLX2 in some developmental contexts (14).

Dlx1 and Dlx2 were hypothesized to be important for ENS development over 20 years ago, but their role in ENS was never carefully evaluated. Dlx2-/− mice die as neonates with massive proximal bowel distention attributed to abnormal motility (10). Unsurprisingly, Dlx1/2-/− mice have a similar phenotype due to a deletion encompassing Dlx1, Dlx2 and intergenic regions. In contrast, Dlx1-/− mice on some genetic backgrounds were reported to die by one month of age (14, 15), a timeframe similar to mouse models with defined enteric neuropathies (12, 16). Consistent with the hypothesis that Dlx1 and Dlx2 mutations affect ENS function or development, both genes are expressed in developing ENS at ages when ENCDCs are migrating, proliferating, and differentiating into neurons and glia, including embryonic days (E) 12.5 (10, 17), E14.5 (18, 19), E17.5 (20), and postnatal day (P)0 (18). Further supporting a role in ENS development, Dlx2 enhances expression of the transcription factor Zfhx1b (also called SIP1 and Zeb2) in CNS (21), and ZFHX1B mutations can cause Hirschsprung disease (a problem where distal bowel lacks ENS) (22, 23). Given compelling evidence implicating DLX1 and DLX2 in ENS development, we were surprised to find no studies thoroughly characterizing ENS in mice lacking these proteins.

To evaluate DLX1 and DLX2’s role in ENS development, we analyzed bowel structure and function in Dlx1/2-/− mice, which die at P0, and Dlx1-/− mice, which survive to adulthood in our colony. We observed serious bowel function defects in Dlx1/2-/− mice at P0, including slower transit and absence of neurally-mediated 4
contractions. Given the critical role of DLX1 and DLX2 in CNS interneuron migration, we initially hypothesized that ENS precursor migration might also be defective in Dlx1/2-/- mice causing Hirschsprung-like disease (absent distal ENS) that explained dysfunctional bowel. To our surprise, ENCDC migration, neuron and glia density, and ratios of neuron subtypes were normal in Dlx1/2-/ mice. To define mechanisms causing defective bowel function, we performed RNA sequencing on E14.5 and P0 Dlx1/2+/+ and Dlx1/2/- ENCDC. We identified dysregulation of many genes, including reduced expression of the neurotransmitter vasoactive intestinal peptide (Vip), and discovered fewer VIP-lineage neurons in Dlx1/2/- mice, which may explain the functional defects in Dlx1/2/- mice. To our knowledge, this study is the first linking Dlx genes to Vip expression.

Results

Dlx1/2-/- and Dlx2-/- mice die as neonates with massive abdominal distention due to intestinal gas accumulation

To evaluate the role of Dlx1 and Dlx2 in bowel development, we bred to generate Dlx1-/-, Dlx2-/-, and Dlx1/2-/- mice. The Dlx1/2-/- mice appeared ill as neonates, lacked a milk spot, and died within 24 hours of birth with massive abdominal distention (Figure 1A-D). Dlx2-/- mice also died early with a similar phenotype to Dlx1/2-/- mice, but survival was not tracked as closely; Dlx2-/- mice were never recovered at weaning (P30). Gross anatomic analysis demonstrated marked accumulation of gas within proximal small bowel (Figure 1E-F). In contrast, Dlx1-/- mice, Dlx2+/+ mice, Dlx1/2+-/- mice and wild type (WT) mice appeared healthy as neonates. Furthermore, although Dlx1-/- mice were
previously reported to grow slowly and die by one month of age (12), we did not observe excess perinatal death of our Dlx1-/- mice (Figure 1G) despite a slow growth trajectory (Figure 1H-I). We note that our Dlx1-/- mice have CreERT2 inserted into the initiation codon, while Qiu et al. deleted Dlx1 exon 2 and 3. Furthermore our mice differed in stain background and mouse colony, which may account for survival differences. To test if Dlx1-/- mice had poor bowel motility, which can cause slow growth, we gavage-fed 37-day-old Dlx1-/- mice with fluorescein isothiocyanate–dextran (FITC-dextran) and evaluated transit through the bowel lumen. FITC-dextran is poorly absorbed across bowel epithelium, and transit of FITC-dextran through the bowel lumen is a well-established method to assess motility (20, 24). We observed no delays in small bowel transit (Figure 1J-K). To assess colon motility, we inserted a glass bead into the distal colon of adult Dlx1-/- and WT mice and timed bead expulsion. We observed a delay in distal colon bead expulsion consistent with dysmotility that did not impact survival (Figure 1L). We noted that Dlx2-/- and Dlx1/2-/- mice have cleft palate as previously reported (10, 12, 25) and that this might cause poor feeding and air swallowing, but the marked accumulation of air in the proximal small bowel suggested that bowel dysmotility slows gas transit. We decided to focus analyses on Dlx1/2-/- mice that lack both DLX1 and DLX2 proteins since some functional redundancy has been reported for Dlx1 and Dlx2 in the CNS.

**Dlx1/2-/- mice have bowel dysmotility**

To determine if bowel dysmotility at P0 contributes to bowel distension in Dlx1/2-/- mice, we fed neonatal mice FITC-dextran and evaluated transit through the
bowel lumen. To ensure conditions for testing were as similar as possible between genotypes, we delivered mice by Cesarean section (C-section) at E19.5 (the usual day of delivery). Cesarean delivery prevented WT and $Dlx1/2^{+/−}$ from feeding and permitted evaluation at a well-defined interval after birth. To delay spontaneous delivery but test bowel transit in full term pups, mothers were treated with progesterone at E18.5. Between 1-3 hours after delivery, each pup received a small bolus of FITC-dextran delivered directly into the oral cavity. All mice swallowed FITC-dextran even when they had cleft palate. Three hours later, pups were euthanized and intraluminal FITC fluorescence intensity was measured for defined bowel regions. In WT and $Dlx1/2^{+/−}$ mice, most FITC-dextran reached mid-small intestine after 3 hours. In contrast, $Dlx1/2^{−/−}$ mutants retained most FITC-dextran in their stomach with small amounts in the proximal small intestine (SI; Figure 2A). A weighted average of fluorescence intensity (i.e., geometric mean) confirmed significant delays in bowel intraluminal transit for newborn $Dlx1/2^{−/−}$ mutant mice (Figure 2B), suggesting abnormal small bowel motility.

Despite our attempts to control for presence of bowel air, many $Dlx1/2^{−/−}$ mice accumulated air in their bowel during the assay. Intriguingly, a subset of $Dlx1/2^{+/−}$ heterozygotes also spontaneously accumulated air in their GI tracts in the absence of cleft palate. Compared to these $Dlx1/2$ heterozygotes with intraluminal air, $Dlx1/2^{−/−}$ mice still had significantly slower FITC-dextran transit (Figure 2C; $p < 0.05$), confirming poor gut motility in $Dlx1/2^{−/−}$ mutant mice.

One possibility was that delayed FITC-dextran transit in $Dlx1/2^{−/−}$ mice could reflect poor health of the $Dlx1/2^{−/−}$ neonates (i.e., ileus as a result of systemic illness). In fact, $Dlx1/2^{−/−}$ mice showed signs of poor health and four $Dlx1/2^{−/−}$ pups died during the
experiment. We excluded these animals and an additional two pups from our analysis due to lack of activity after the 3 hour study was complete. Dlx1/2-/- pups included in the experiment were more likely to be pale-appearing and exhibited fewer movements in the first few hours of life (Supplementary Figure 1A-B). These concerns prompted us to perform an in vitro motility assay to complement our in vivo studies. We again treated dams with progesterone and delivered pups via Cesarean section to control for food and air in the bowel. Bowel was carefully removed from neonates shortly after delivery and was placed in warmed, oxygenated Krebs-Ringers solution in a continuously-perfusing organ bath. Small intestine was cannulated at the proximal end and perfused with oxygenated Krebs-Ringers using an oral pressure of 1.3 cm H2O above the bowel lumen. Videos of bowel contractions were recorded in the presence or absence of tetrodotoxin (TTX; see Supplementary Videos 1-2 for representative videos). Videos were converted to spatiotemporal maps or "kymographs" depicting bowel width (color axis) over time (y-axis) for the first 2 cm of SI (Figure 2D-G). In control bowel, we observed low-frequency (~0.4 contractions/minute) and high-frequency (6-18 contractions/minute) contractions as has been previously described in neonatal mice (26). The low-frequency contraction complexes that typically occurred at regular intervals (i.e. rhythmically) in control mice were abrogated by TTX (Figure 2H), indicating neuronal origin. The Dlx1/2-/- mice had many fewer low-frequency contraction complexes than controls (p=0.0055; Figure 2H) and none of the contraction complexes in Dlx1/2-/- mouse small bowel occurred in a rhythmic pattern (Figure 2I). This analysis confirmed that neurally-mediated motility patterns are abnormal in the small bowel of Dlx1/2-/- mice.
Unlike low-frequency contractions, high-frequency contractions were present and appeared similar in controls (Dlx1/2+/+ and Dlx1/2+/-) and Dlx1/2-/ - SI. These more frequent contractions are probably mediated by interstitial cells of Cajal or smooth muscle since they are TTX insensitive (26). Furthermore, Fourier transform analysis of the frequency of these faster contractions (Figure 2J) as described (26) showed no difference between control and Dlx1/2-/ - mutant bowel (Figure 2K), consistent with neuropathic origin of Dlx1/2-/ - bowel dysmotility.

We next tested if small bowel in Dlx1/2-/ - mice was tonically contracted or dilated, problems that might suggest an imbalance in excitatory or inhibitory motor neuron signaling and could slow bowel intraluminal transit. Our analysis showed that Dlx1/2-/ - bowel was neither more dilated nor more contracted than control bowel (Figure 2L). Baseline tortuosity (measured as length of pinned bowel divided by Euclidean distance from end to end in randomly-selected video frames) was also no different in Dlx1/2-/ - bowel (Figure 2M). Control bowel became more contracted and tortuous on exposure to TTX (Figure 2L and 2M), suggesting that neural activity is necessary for baseline bowel relaxation in P0 SI. Dlx1/2-/ - bowel did not become significantly more contracted in the presence of TTX (p=0.1423), but became more tortuous with TTX application (p=0.0276), consistent with a distinct pattern of neuromodulation in Dlx1/2-/ - mutant bowel (Figure 2L and 2M). Collectively, these in vitro and in vivo data suggest that bowel motility is profoundly abnormal in Dlx1/2-/ - mice and that dysmotility is due to defects in neuronal activity.
Dlx1 and Dlx2 are not necessary for bowel colonization by ENCDC during fetal development

Because bowel motility was abnormal in Dlx1/2-/- mice, we tested the hypothesis that DLX1 and DLX2 influence colonization of the bowel by ENCDC during fetal development. We analyzed bowel at E12.5 when ENCDC have normally colonized the entire SI and half the colon. The extent of ENS precursor migration was assessed using an antibody to neuron-specific class III β-tubulin (TuJ1) which is expressed at high levels in enteric neurons including at the migration wavefront (27, 28). By this criterion, E12.5 Dlx1/2/-/- mice had normal colonization by ENCDC (Figure 3A-C). In contrast, Dlx2-/- mice had a small reduction in bowel colonization by ENCDC of dubious functional significance and marginal statistical significance (66.5 ± 1.5% colonized (+/+)) vs 47.4 ± 5.2% colonized (-/-); p = 0.0499; Figure 3E-G), perhaps reflecting relatively protective CD1 strain background for Dlx1/2 compared to aganglionosis promoting C57BL/6J strain background (29) for Dlx2-/- mice. Because some gene defects are only apparent with a sensitized genetic background (30), we next evaluated bowel colonization by Dlx1/2-/- ENCDC in the context of Ret heterozygosity. Dlx1/2-/-;Ret+/- mice on a mixed CD1 x C57Bl6/J background, had apparently normal colonization of the bowel by ENCDC (Figure 3I-K). Colon length was also normal in all mutant mice (Figure 3D, H, L). These observations suggest that bowel dysmotility in Dlx1/2-/- mice is not due to abnormal bowel colonization by ENCDC.

To determine if Dlx1/2-/- mice have major ENS structural defects causing dysmotility, we dissected mutant mice just after birth (P0) and stained bowel whole mounts with antibodies against the neuronal RNA-binding protein HuC/D (ANNA-1),
neuron-specific class III β-tubulin (TuJ1), SOX10 (which is present in ENS precursors and mature glia), and the glia-specific marker S100β. There were no obvious differences in ENS organization or staining patterns between $Dlx1/2^+/+$ and $Dlx1/2^-/-$ mice. TuJ1 staining showed normal organization of nerve fibers. Quantitative analysis of HuC/D staining demonstrated normal neuron density in proximal SI, distal SI, and colon of P0 $Dlx1/2^-/-$ mice (Figure 4A-O). For small intestine, we assessed neuron density in both myenteric and submucosal plexus, but due to difficulty separating muscle from submucosa in neonatal mice, we were only able to assess total neuron density (myenteric plexus + submucosal plexus) in colon. P0 $Dlx1/2^-/-$ mice also had normal SOX10+ cell density and SOX10+S100β+ glia density in bowel regions examined (Figure 4P-W).

Since neuron death occurs in subsets of CNS neurons in $Dlx1^-/-$ mice postnatally (15), and since we observed slightly delayed colon motility, we assessed neuron numbers in 10-week old $Dlx1^-/-$ mice, which lack cleft palate and survive to adulthood. We found no differences in neuron density in proximal SI, distal SI, mid-colon, or distal colon in $Dlx1^-/-$ adults in either myenteric or submucosal plexus (Figure 5A-X). We also found no difference in SOX10+ glia and ENS precursors in distal SI of mature $Dlx1^-/-$ mice (Figure 5Y-AA).

No defects in ENS subtype ratios were detected in $Dlx1/2^-/-$ and $Dlx1^-/-$ mice

The ENS has over twenty neuron subtypes that perform distinct functions (31). Because total neuron numbers are normal in $Dlx1/2^-/-$ mice, but bowel motility is abnormal, we hypothesized DLX1 and DLX2 might be required for differentiation or survival of one or more neuron subtypes, leading to dysmotility in $Dlx1/2^-/-$ mice. To test
this hypothesis, we stained P0 bowel with antibodies that distinguish many types of enteric neuron. We observed no differences in ratios of neurons expressing myenteric plexus neurotransmitter markers nitric oxide synthase (NOS), calretinin, γ-aminobutyric acid (GABA), or vasoactive intestinal peptide (VIP; Figure 6A-E, G-K, M-Q) or the submucosal plexus neurotransmitter VIP (Figure 6F, L, R). Since we lacked reliable antibodies for choline acetyl-transferase (ChAT), we bred and analyzed Dlx1/2;ChAT-EGFP-L10a mice but found no difference in EGFP+ neuron number (Figure 6A, G, M; Supplementary Figure 2A-N). One technical issue is that fluorescence intensity of immunohistochemical signals for certain neurotransmitters at P0 (substance P, tyrosine hydroxylase) was quite low (Supplementary Figure 3A-D). We suspect this reflects incomplete differentiation of these subtypes at P0, since immunostaining in adult mouse ENS was excellent for all antibodies used (Supplementary Figure 3E-J). Despite substantial effort, we were unable to identify neuron populations that were dramatically altered at an anatomic level to explain the severe intestinal dysmotility in neonatal Dlx1/2-/− mice.

In Dlx1-/− mice, we evaluated ENS in adult bowel since somatosensory cortex of the CNS has normal neuron numbers at birth, but postnatal apoptosis occurs in several cortical neuron subtypes, including GAD67+ (which catalyzes GABA synthesis from glutamate), NOS+, somatostatin+, and calretinin+ neurons (15). Taking advantage of postnatal survival in Dlx1-/− mice, we determined if similar age-dependent neuronal subtype loss occurs in the Dlx1-/− ENS by staining adult Dlx1-/− distal SI with antibodies marking NOS, GABA, somatostatin, and VIP. Neuron subtype ratios were similar in WT
and Dlx1/- mice (Figure 6S-DD) suggesting that post-natal enteric neuron loss is not occurring.

**Vip is dysregulated in E14.5 and P0 Dlx1/2/- ENCDC**

Since our detailed anatomic analyses failed to reveal obvious structural problems of the ENS of Dlx1/2/- mice, we next hypothesized that dysmotility in Dlx1/2/- mice is due to altered gene expression not discernable at the level of ENS anatomy. We tested this hypothesis by RNA-seq of pooled ENCDC isolated from E14.5 and P0 bowel using fluorescence activated cell sorting (FACS). We chose these ages for theoretical and technical reasons. E14.5 was selected because Dlx1 and Dlx2 are expressed in murine ENS by E12.5 (10, 17), and we wanted to evaluate early effects of mutations, rather than secondary effects that occur as development proceeds. Furthermore, at E14.5 there are many ENCDC for analysis, and neuron subtype markers are starting to appear as enteric neural networks are established. We also analyzed P0 ENS to match our functional experiments performed using neonatal bowel. For our E14.5 analysis, we bred Dlx1/2 mice to an Ednrb-L10A-GFP reporter line that expresses a fluorescent protein in ENCDC at E14.5 (32) and performed RNA-seq on FACS-sorted ENCDC from E14.5 Dlx1/2-/-;Ednrb-L10A-GFP<sup>Gfp/wt</sup> (mutant) and Dlx1/2+/-;Ednrb-L10A-GFP<sup>Gfp/wt</sup> (WT) SI. To analyze P0 bowel, we bred Dlx1/2 mice to a Wnt1-Cre;R26R-Tdtomato line and performed RNA-seq on FACS-sorted ENS from P0 Dlx1/2-/-;Wnt1-Cre<sup>cre/wt</sup>;R26R-Tdtomato+ (mutant) and Dlx1/2+/-;Wnt1-Cre<sup>cre/wt</sup>;R26R-Tdtomato+ (WT) SI.
We identified 22 dysregulated genes in Dlx1/2-/ E14.5 ENCDC using q < 0.1 as a statistical threshold after filtering out genes with low expression (average expression < 1) and fold change (|log2 fold change| < 1; Figure 7A-B). Given that DLX2 regulates Zfhx1b in CNS, we had hypothesized that Zfhx1b might be dysregulated in the Dlx1/2-/ ENS, but we found no evidence of altered Zfhx1b levels. Remarkably, however, we observed ~84% reduction in mRNA levels for the ENS neurotransmitter vasoactive intestinal peptide (Vip) and a 3-fold increase in abundance of mRNA for the neurotransmitter proenkephalin (Penk) in small bowel ENCDC from Dlx1/2-/ mice compared to WT animals. We also observed changes in receptors (Lifr), extracellular matrix genes (Mmp2, Col3a1), cytoskeletal regulators (Dnm3, Capn6, Fmn1), neurite growth factors (Ptn), and glia-associated genes (Plp1, Lrp1b; Figure 7B). Since neurotransmitter dysregulation might most directly explain bowel dysmotility in Dlx1/2-/ mice, we attempted to validate altered levels of Vip and Penk mRNA using quantitative RT-PCR on independently isolated E14.5 mutant and control samples (Figure 7C-D; see Supplementary Figure 4 for standard curves). qRT-PCR analyses showed 3-fold reduction of Vip mRNA in E14.5 Dlx1/2/- ENCDC compared to WT (1.0 ± 0.11 (+/+)) vs 0.36 ± 0.10 (-/-), p = 0.004) and no statistical difference in Penk mRNA (1.0 ± 0.02 (+/+)) vs 1.34 ± 0.17 (-/-), p = 0.063). Although we identified fewer dysregulated genes at P0, our RNA-seq data suggest Vip may continue to be dysregulated in Dlx1/2/- neonatal ENS (Figure 7E-F).

To further explore the VIP-expressing enteric neuron subpopulations, we bred to generate Dlx1/2;Vip-IRES-Cre;R26R-TdTomato mice, which produce TdTomato in cells expressing Vip. Although we had already counted VIP immunoreactive enteric neurons,
VIP is primarily in neurites, making cell counting suboptimal. We discovered that TdTomato+ neurons in mid-SI are significantly reduced in absolute cell density (18.4%) and relative to total neuron number (13.2%) in myenteric plexus of Dlx1/2;Vip-IRES-Cre;R26R-TdTomato mice (Figure 8A-D). Furthermore VIP reporter (TdTomato+) neuron density was dramatically reduced in absolute (51.5%) and relative terms (41.0%) in the submucosal plexus of Dlx1/2;Vip-IRES-Cre;R26R-TdTomato mice (Figure 8F-I). As before, we saw no difference in total neuron density (Figure 8E, J). We also confirmed that most TdTomato+ neurons appeared to express VIP protein at P0 (Supplementary Figure 5A-C).

Dlx1/2-/- mice on a mixed background have fewer circular smooth muscle-projecting neurites

To determine if VIPergic neurites differed in mutants, we immunostained Dlx1/2;Vip-IRES-Cre;R26R-TdTomato SI with an antibody against neurites (TuJ1). We first examined nerve fibers within the myenteric plexus. All large nerve fiber bundles were TdTomato+ (Figure 9A-H). We found no difference in nerve fiber bundle diameter between Dlx1/2-/- mice and controls (Figure 9I-J).

To determine if the relative density of VIP+ neurites projecting into circular smooth muscle (CSM) is altered in Dlx1/2-/- mice, we quantified neurite density by drawing a line perpendicular to the axis of CSM neurite projections and counting thin fibers crossing that line. Total density of CSM TuJ1+ neurites was significantly reduced in Dlx1/2-/- mice (22.3% reduction, p=0.0077; Figure 9K-Q). However, the ratio of TdTomato+ neurites to total was not reduced (Figure 9R). TdTomato+ neurite density did
not reach our statistically threshold, but mean neuron density was also reduced (p=0.1006; Figure 9S). To determine if the altered neurite density was consistent across strain backgrounds, we quantified density of proximal SI CSM-projecting TuJ1+ neurites in *Dlx1/2-/-* mice on a CD1 background, but we found no difference in neurite density between mutants and controls (p=0.3193; Figure 9T). These data suggest *Dlx1/2* may affect targeting or growth of some neurites in CSM.

To further investigate how *Dlx1* and *Dlx2* affect neurite growth, we cultured ENCDCs from unselected E12.5 bowel in the presence of glial cell-line derived neurotrophic factor (GDNF) and labeled neurites using TuJ1. For this experiment, we used *Dlx1/2;ChAT-EGFP-L10a* mice on a mixed CD1xC57BL/6 background, because we initially hoped to use EGFP to distinguish ChAT+ and ChAT- neurons in culture; however, EGFP was not expressed at this young age. Cultured neurons exhibited highly variable morphology (Supplementary Figure 6A-B). Quantification of total neurite length per cell body revealed no obvious differences between *Dlx1/2-/-* and WT neurons (Supplementary Figure 6C; >400 cells traced), and histograms of total neurite length for individual traced cells were similar for mutant and wild type neurons (Supplementary Figure 6D-E).

**Dlx1/2-/- mice have reduced VIP in myenteric plexus nerve fibers and soma**

To determine if VIP is reduced in myenteric nerve fibers, we immunostained P0 mouse SI with antibodies against TuJ1, HuC/D, and VIP and imaged at high power. Using CellProfiler image analysis software, we created a mask defined by TuJ1, but not HuC/D (i.e. myenteric plexus nerve fibers, with cell bodies excluded) and computed 16
average VIP intensity within the masked region (Figure 10A-K). Average VIP intensity within myenteric plexus nerve fibers was significantly reduced in Dlx1/2-/- mice relative to WT (Figure 10K), demonstrating dramatic reductions in VIP protein in myenteric plexus nerve fibers. Moreover, the relative area of nerve fiber bundles with high VIP expression (likely representing concentrated collections of VIP, i.e. varicosities) was even more dramatically decreased in Dlx1/2-/- mice (Figure 10L-N).

We used similar methods to determine if VIP within nerve cell bodies was reduced. Average VIP intensity within soma was not significantly changed in Dlx1/2-/- mice (Figure 10O-Q). However, the relative area of soma with high VIP expression was significantly decreased in Dlx1/2-/- mice (Figure 10R-T). Together, these results confirm reduced VIP protein in Dlx1/2-/- ENS.

**Discussion**

Bowel motility disorders include life-threatening problems like Hirschsprung disease where the ENS is missing from distal bowel (33) and neuropathic CIPO where ENS is present, but dysfunctional (1). Human CIPO causes abdominal distension and bowel dysmotility that resembles bowel problems in Dlx1/2-/- mice, but anatomic defects are often difficult to identify in CIPO. While it is possible that more careful analysis of ENS structure would identify defects in human CIPO bowel, functional defects can also occur in the absence of anatomic abnormalities. For Dlx1/2-/- mice, we did, eventually uncover anatomic defects, but only after RNA-seq identified reduced Vip levels in mutant ENS. RNA-seq is possible in human ENS, and might also be valuable in analysis of bowel from people with CIPO (34, 35).
We chose to investigate bowel motility in Dlx1 and Dlx1/2 mutants because Dlx1 and Dlx2 are expressed in developing ENS at ages relevant for ENCDC migration, differentiation, and subtype specification, and because the original description of Dlx2-/- mice suggested they die from bowel dysmotility (10, 17-20). We showed that neonatal Dlx1/2-/- mice have profound bowel function defects, including delayed gastric emptying and slow small bowel transit via an in vivo FITC-dextran assay. FITC-dextran was found in the stomachs of all Dlx1/2-/- mice tested, confirming that despite cleft palate, Dlx1/2-/- mutants were capable of swallowing and that stomach empties slowly. When we maintained bowel in an oxygenated organ bath and treated with TTX, which blocks nerve cell activity, we found Dlx1/2-/- bowels had severely reduced TTX-sensitive contraction patterns. Interpreting these findings is challenging because a complex interplay of excitatory and inhibitory neural input regulates bowel smooth muscle contraction and relaxation. A naïve interpretation is that neuron-mediated smooth muscle excitation (contraction) is defective in Dlx1/2-/- mice. Another possibility is that Dlx1/2-/- bowel has reduced smooth muscle inhibition at baseline, leading to a mildly contracted phenotype approaching that of control bowel treated with TTX. Consistent with this hypothesis, Dlx1/2-/- bowel did not seem to contract significantly upon TTX treatment, unlike control bowel. Moreover, bowel videos suggest that neurally-mediated "contraction complexes" actually consist of complex motor patterns, where bowel first straightens (possibly reflecting longitudinal smooth muscle relaxation), then contracts rapidly, and finally returns to baseline tortuosity (Supplementary Video 1). TTX-treated bowel, which is highly contracted, never undergoes this pattern, and Dlx1/2-/- mouse bowel does so only rarely. Since Dlx1/2-/- mice had a normal pattern of TTX-insensitive 18
(i.e. non-neuronal) contractions, and $Dlx1$ and $Dlx2$ are prominently expressed in developing ENS, we interpret these findings to mean that $Dlx1/2$ mutations cause neuropathic CIPO-like disease in mice.

Surprisingly, immunohistochemical studies showed $Dlx1/2$-/- bowel had largely normal ENS structure at birth, with normal neuron and glia density and normal ratios of many enteric neuron subtypes, even though changes in these parameters have been identified in many mouse lines (3, 36-42). We note, however, that our extensive anatomic studies were not exhaustive, that some antibodies gave weak signals in P0 ENS (e.g., tyrosine hydroxylase, substance P), and that we did not perform enough replicates to define sex-restricted defects. Furthermore, although $Dlx2$-/- mice had a small ENCDC migration delay at E12.5, this may occur because of different strain backgrounds for $Dlx2$-/- (C57BL/6) and $Dlx1/2$-/- mice (CD1). Strain effects on ENCDC migration have been reported in the ENS (29). It is, however, unlikely that this minimal transient ENCDC migration delay impairs bowel function at P0.

To identify defects causing CIPO-like disease in $Dlx$ mutant mice, we performed RNA-seq. Unexpectedly, we identified a novel connection between $Dlx$ genes and $Vip$.

The $Dlx1/2$-/- ENS had decreased $Vip$ mRNA at P0 (53% reduction) and a greater reduction in $Vip$ mRNA at E14.5 (64% reduction by qPCR, 84% reduction by RNA-seq). Consistent with RNA data, the intensity of VIP immunostaining in cell bodies and in neurites was significantly reduced in $Dlx1/2$-/- myenteric plexus. Moreover, $Dlx1/2$-/-;VIP-ires-Cre;TdTomato reporter mice had significantly fewer TdTomato+ neurons in myenteric (18.4%) and submucosal plexus (51.5%), even though we could not appreciate neuron loss via VIP immunohistochemistry. We attribute this difference to limited VIP
protein in nerve cell bodies, which may make neuron counting less reliable with antibody
staining than using Vip-lineage reporter mice.

In the ENS, VIP acts as a critical ENS neurotransmitter with multiple roles. In
myenteric plexus, VIP is co-expressed with nitric oxide synthase and PACAP in
inhibitory motor neurons. These neurons inhibit the multicellular motor syncytium
composed of smooth muscle cells (SMC), interstitial cells of Cajal (ICC), and platelet-
derived growth factor receptor (PDGFR)α+ cells (5). In submucosal plexus, VIP+
secretomotor and vasodilator neurons influence epithelial secretion and blood flow,
respectively. VIP also acts on neurons and glia within the ENS (43) and is an important
modulator of gut immunity (44). Thus, there are many ways that altered VIP abundance
might impact gut motility, epithelial function and immune system activity to impair
Dlx1/2-/− mouse survival. The importance of VIP in ENS function is highlighted by the
observation that Vip-/− mice have severely delayed small intestine transit, impaired mucin
production, smooth muscle thickening, shortened bowel, and die early due to GI stenosis
(45). In contrast, Vip+/− mice are healthy enough to reproduce, but limited data exists
about bowel motility in Vip heterozygotes. These observations are consistent with the
hypothesis that reduced VIP levels in Dlx1/2-/− mice could contribute to bowel
dysmotility, but it is not easy to determine if the level of VIP reduction detected in
Dlx1/2-/− mice adequately explains the profound bowel motility defects in these animals.
We found altered levels of many additional mRNA in the ENS of Dlx1/2-/− mice and
expression changes in one or more of these genes might also contribute to bowel
dysmotility.
Our findings highlight striking differences between the role of *Dlx1* and *Dlx2* in the ENS and their developmental functions in the brain. In CNS, *Dlx1* and *Dlx2* are required for migration of multiple populations of inhibitory interneuron precursors from the ventral telencephalon into the neocortex and olfactory bulb (13, 14, 46). *Dlx2* also regulates expression of the transcription factor *Zfhx1b* in brain (21). *Zfhx1b* is critical for normal ENCDC migration, and in humans *ZFHX1B* mutations cause Mowat-Wilson syndrome (a genetic disorder characterized by intellectual disability, epilepsy, microcephaly, and Hirschsprung disease) (22, 30, 47). Given this substantial evidence implicating *Dlx1* and *Dlx2* in processes critical for neuron migration and differentiation, we were surprised that *Dlx1/2-/-* bowel exhibited normal ENCDC migration at E12.5 and normal neuron density at P0.

In CNS, *Dlx1* and *Dlx2* are necessary for expression of glutamic acid decarboxylase 1 and 2 (*Gad1* and *Gad2*), which catalyze gamma-amino butyric acid (GABA) synthesis from glutamate (48, 49). Surprisingly, we found normal numbers of GABAergic neurons in *Dlx1/2-/-* ENS, and we did not observe significantly decreased *Gad1* or *Gad2* gene expression in *Dlx* mutant ENCDCs at E14.5 or P0. These findings suggest that in the ENS, GABAergic neurons do not depend on *Dlx1* or *Dlx2* for *Gad1*, *Gad2* or GABA synthesis. (Interestingly, our GABA antibody labeled more neurons than were previously reported in the literature (53% vs 5-8% of myenteric neurons) (40, 41). We suspect this is because we quantified both brightly- (~11%) and dimly-labeled (~42%) populations of GABA+ neurons (Figure 6X), whereas prior studies may have only quantified brightly stained neurons). We also note that loss of *Dlx1* causes apoptosis in subsets of CNS interneurons in 1-2-month-old mice (15), but *Dlx1* adult ENS showed...
no evidence of neuron subtype loss or decreased neuron density. Together, these findings underscore highly distinct roles for $Dlx$ genes in enteric neurons, compared to the CNS.

Summary: Chronic intestinal pseudo-obstruction (CIPO) describes a poorly-understood constellation of diseases in which bowel motility is abnormal despite the presence of neurons throughout the bowel. Although neuron dysfunction accounts for many cases of CIPO, few causative genes have been identified (1, 5, 50). Our study provides a unique example of how bowel motility defects may occur in the absence of easily identified ENS structural defects, likely due to gene-level dysregulation. To our knowledge, few prior studies have performed such a comprehensive analysis of the neonatal ENS in mutant mice. There is a strong need to develop new tools to assess motility at young ages, since many ENS-relevant genes are also critical for palate development (12, 20, 51), kidney development (52-54), and other systems essential for life after birth. Our study is also the first to comprehensively investigate the role of DLX1 and DLX2 in gut motility, and the first to show a regulatory link between DLX1 and DLX2 and $Vip$. Remarkably, well-described functions for $Dlx1$ and $Dlx2$ in CNS like regulation of $Gad1$ and $Gad2$ to make GABA and control of cell migration do not appear to occur in the ENS. Furthermore, DLX2 was reported to be essential for CNS $Zfhx1b$ expression, and mutations in $Zfhx1b$ lead to almost complete loss of enteric neurons in mice, whereas bowel colonization by ENCDC proceeds normally in $Dlx1/2^{-/-}$ mice. Collectively these observations highlight how differently genes shared between ENS and CNS may function. Future studies in humans are important to determine if $Dlx$ mutations are present in individuals with CIPO and to elucidate if $Dlx$ genes control $Vip$ expression in developing human ENS.
Methods

Animals

All mouse experiments were performed in accordance with the Children’s Hospital of Philadelphia Institutional Animal Care and Use Committee. Del(Dlx1-Dlx2)^{1/Jlr}/Mmucd mice and B6.129X1-Dlx2^tm1/Jlr/Mmucd mice (referred to as Dlx1/2 and Dlx2 mice respectively) (10, 12) were obtained from the MMRRC (RRID:MMRRC_036673-UCD and RRID:MMRRC_015870-UCD) and were maintained on a CD1 or C57BL/6J background, respectively. B6;129S4-Dlx1tm1(cre/ERT2)Zjh/J (referred to as Dlx1-CreERT2) mice were ordered from The Jackson Laboratory (RRID:IMSR_JAX:014551) and were maintained on a C57BL/6J background. EDNRB-EGFP-L10a mice (32) were bred into Dlx1/2 mice and maintained on a mixed C57BL6/J X CD1 background. ChAT-EGFP-L10a mice (RRID:IMSR_JAX:030250; C57BL/6J) were a kind gift from Joseph Dougherty at Washington University School of Medicine in St. Louis. Dlx1/2;ChAT-EGFP-L10a mice were obtained by breeding homozygous ChAT-EGFP-L10a mice to Dlx1/2 heterozygotes and were maintained on a mixed C57BL/6J x CD1 background. Dlx1/2;Ret mice were generated by breeding Dlx1/2 mice to Ret^{TGM} mice (referred to as Ret mice; C57BL/6J) (55) and were maintained on a C57BL/6J x CD1 background.

Tg(Wnt1cre)11Rth mice (referred to as Wnt1-Cre; RRID:IMSR_JAX:003829) and Gt(Rosa)26Sortm9(CAGtdTomato)Hze mice (referred to as R26R-TdTomato; RRID:IMSR_JAX:007909) were obtained from The Jackson Laboratory (Bar Harbor, ME). Wnt1-Cre; R26R-TdTomato mice on a mixed C57BL/6J x CBA/J)F1 background were bred into Dlx1/2 mice and maintained on a mixed C57BL/6J x CD1 x CBA/J)F1 background.
Vip\textsuperscript{tm1(cre)Zjh/J} mice (referred to as \textit{Vip-IRES-Cre}; RRID:IMSR_JAX:010908) on a mixed C57BL/6;129S4 background were bred into \textit{Dlx1/2} and \textit{R26R-TdTomato} mice and maintained on a mixed C57BL/6J x CD1 x 129S4 background. Genotyping was performed using previously published and novel primers (Supplementary Table 1). Vaginal plug day was considered E0.5.

**Preparation of whole gut samples**

Adult mice were euthanized in CO\textsubscript{2} (5 minutes) and cervically dislocated. P0 mice were euthanized by decapitation. Bowel was placed in cold PBS, opened along the mesenteric border, and pinned onto Sylgard\textsuperscript{®} 184 Silicone Elastomer (Dow Corning, Midland, MI), serosa up, using insect pins. Tissue was fixed in 4\% paraformaldehyde (20-30 minutes, 25°C) and transferred to PBS. Muscle was peeled from the mucosa and submucosa to separate myenteric and submucosal plexus. Peeled gut was equilibrated at room temperature for 30 minutes in 50\% glycerol/50\% PBS and stored (-20°C) until staining.

To prepare E12.5 bowel, pregnant dams were euthanized in CO\textsubscript{2} (5 minutes) and embryos dissected in Leibovitz’s L-15 medium (ThermoFisher, Waltham, MA; Cat# 41300039). Bowel was fixed in 4\% paraformaldehyde (30 minutes), equilibrated at room temperature (30 minutes) in 50\% glycerol/50\% PBS, and stored (-20°C) until staining.

**E12.5 Neuron Culture**

Pregnant dams were euthanized (CO\textsubscript{2}, 5 minutes) and E12.5 embryos were dissected in Leibovitz’s L-15 medium. Whole bowel was incubated in dispase (0.2 mg/mL; ThermoFisher, Cat#17105-041) and collagenase (0.2 mg/mL; Sigma-Aldrich, St. Louis, 24
MO; Cat# C-6885) in PBS (5 minutes, 37°C) was rinsed with cold PBS using a cell
strainer, dissociated by triturating 35x through a P1000 pipet, then centrifuged and
resuspended in Neurobasal Media (Invitrogen, Carlsbad, CA; Cat# 21103049)
supplemented with L-glutamine (2 mM; Invitrogen, Cat# 25030081), B27 (1X;
Invitrogen, Cat# 12587010), penicillin/streptomycin (1X; Invitrogen, Cat# 15140122),
and 50 ng/mL 6XHis-GDNF prepared as described (56). Cells were plated on Nunc™
Lab-Tek™ 8-well chamber slides (ThermoFisher, Cat# 177402) coated overnight with
100 µg/mL poly-D-lysine (Sigma, Cat# P7280) and 20 µg/mL laminin (VWR, Cat#
47743-682) for 2hr. After two days, cultures were fixed (15 minutes, 4%
paraformaldehyde) and immunostained.

**Immunofluorescent and enzymatic staining of whole mount**
Whole mount bowel stored in 50% glycerol/50% PBS at -20°C was rinsed in PBS. Fixed
E12.5 bowel was maintained in 48-well plates for the duration of immunostaining.
Samples were blocked (2 hours) in PBS + 0.1% or 0.5% Triton X-100 (PBST) with 5%
Normal Donkey Serum (NDS; Jackson Immuno Research Laboratory, West Grove, PA)
and incubated in primary antibody with gentle rocking at 4°C overnight, except for
HuC/D (ANNA-1) antibody, where we incubated tissues for two hours at room
temperature. Primary antibodies are in Supplementary Table 2. Tissues were washed 3x5
minutes in PBST and incubated in secondary antibody (Supplementary Table 2) at room
temperature with gentle rocking (1 hour). After 3 additional 5-minute washes in PBS,
samples were mounted serosal side up in 50% glycerol/50% PBS on glass slides.
NADPH diaphorase staining was performed as described (57). Briefly, NADPH (Sigma-
Aldrich) and Nitro Blue Tetrazolium were dissolved in PBS with 0.2% Triton-X-100. Samples were incubated in this solution (7-15 minutes, 37°C), rinsed in PBS, then stained with antibodies as described above. For tissue labeled with ChAT antibody, samples were fixed in 100% methanol on ice (30 min) and placed in Dent's bleach (1:4:1 100% DMSO: 100% Methanol: 30% H₂O₂) for 1 hr at RT prior to blocking.

Immunofluorescent staining of cultured cells

Slides were rinsed in PBS, blocked (1 hour in PBS + 0.1% or 0.5% Triton X-100 (PBST) with 5% NDS), and incubated with primary antibody (Supplementary Table 2) overnight (4°C). Tissues were washed 3x5 minutes in PBS and incubated in secondary antibody (Supplementary Table 2) for 1 hour (RT). After 3 additional 5-minute washes, samples were mounted in 50% glycerol/50% PBS + DAPI or Vectashield.

Microscopy

Zeiss Axio Imager.A2, Axio Observer.A1, or LSM 710 microscopes and Zeiss Zen software were used to acquire images. Confocal images show single optical projections or maximum intensity projections as indicated in figure legend. For image processing, ImageJ and Photoshop were used to rotate, crop, and uniformly color adjust images.

Quantitative analysis of antibody-stained bowel

Unless otherwise specified, 5-10 randomly selected 20x fields (a 0.045 mm² region) per sample were analyzed for all quantifications. Using whole mount stained samples, we counted the number of enteric neurons (HuC/D+ cells), enteric glia (SOX10+S100β+) and...
cells), and SOX10+ cells (a mix of glia and ENS precursors) to determine density.

Enteric neuron subtype proportions were quantified by counting the number of HuC/D+, GFP+, nNOS+, calretinin+, NADPH-diaphorase+, GABA+, VIP+, or somatostatin+ cell.

Neurite fiber density was quantified drawing a line perpendicular to circular smooth muscle and counting the number of neurites that crossed the line. Observers were blinded to genotype for all quantitative analyses.

For measuring VIP intensity, we used 5-10 randomly selected 63x confocal z-stacks per sample. Only slices capable of being accurately thresholded were analyzed. Using CellProfiler (58), images were thresholded with the Global with Minimum Cross Entropy settings and masked to quantify VIP intensity within HuC/D+ cell bodies and in HuC/D-TuJ1+ nerve fibers.

Quantitative Analysis of Neurites in Culture

One large (>12 mm²) image was taken within a well (2 separate wells per embryo).

Neurites from intact cells were traced, and cell bodies were counted using the Simple Neurite Tracer plugin in ImageJ (NIH) by investigators blinded to genotype. Because neurites sometimes crossed making tracing difficult, we determined average total neurite length per cell by dividing the sum of all neurite lengths by the number of nerve cell bodies. When neurites could be traced unambiguously, we measured the length of all neurites, added these lengths together to determine total neurite length, and plotted data in a histogram.

Delivery by Cesarean Section
Pregnant mouse mothers (E18.5) were injected subcutaneously with 2 mg of progesterone (Sigma-Aldrich, #P3972-5G). The next morning (E19.5), mice were euthanized via cervical dislocation. Pups were removed from the mother as previously described (59) and placed on a warming pad (DCT-20, Kent Scientific, Torrington, CT).

**Intestinal Transit Assay**

**P0:** A P0 FITC dextran intestinal transit assay was performed as previously described (60), with two modifications: (1) a total of 3 hours passed between time of feeding and time of dissection, and (2) bowel was cut into 10 segments (esophagus, stomach, 6 SI pieces, cecum, and colon).

**P35:** P35 mice were food-deprived 14 hours and gavage-fed 100uL of 10 mg/mL FITC-Dextran (70 kiloDalton) plus 2% methylcellulose. After 1 hour, mice were euthanized (CO₂) and whole bowel was dissected, cut into 16 segments (stomach, 10 SI, 2 cecum, and 3 colon pieces), and placed in Eppendorf® tubes containing 400 uL PBS. Tubes were vortexed and centrifuged (4000 RCF, 10 minutes). 50 uL of supernatant was combined with 50 uL of PBS and analyzed using a fluorometer.

**RNA extraction**

E14.5 dams were euthanized with CO₂. P0 pups were delivered by Cesarean section as described above. Pups were rapidly removed from the mother. E14.5 small intestine was dissected in ice-cold Leibovitz's L-15 medium (Life Technologies, Cat# 41300039) and dissociated (15 minutes, 37°C) in dispase (0.2 mg/mL; ThermoFisher, Cat# 17105-041).
and collagenase (0.2 mg/mL; Sigma, Cat# C-6885) in PBS with P1000 trituration. P0 small intestine was dissected in Leibovitz's L15 medium, snipped into small pieces using insulin needles (Beckton Dickerson, Cat# 08290-3284-18), and dissociated (30 minutes, 37°C) in Liberase (Sigma-Aldrich, Cat# 5401135001) supplemented with DNase I (Roche, Basel, Switzerland; Cat# 04716728001), MgCl₂ (6 mM) and CaCl₂ (1 mM) in HBSS with repeated P1000 trituration. Fluorescent EGFP-L10a⁺ cells (E14.5) or Tdtomato⁺ cells (P0) were sorted on a BD FACSJazz and collected in Neurobasal medium (Invitrogen, Cat# 21103049). RNA was extracted using the Qiagen RNeasy Plus Micro kit (Qiagen, Hilden, Germany; Cat# 74034) combined with Qiagen's RNase Free DNase Set (Qiagen, Cat# 79254). Samples were run on an Agilent Bioanalyzer and used if RNA Integrity Number (RIN) was > 7.0.

Bowel Physiology

Between 1-3 hours after Cesarean section, pups were decapitated. Bowel was removed and placed in warmed (37°C), oxygenated (95% O₂, 5% CO₂) Krebs-Ringers solution (118 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1 mM NaH₂PO₄, 25 mM NaHCO₃, 11 mM D-Glucose, pH 7.4) in a horizontal organ bath (Hugo Sachs Elektronik Harvard Apparatus, March, Germany; Cat# D-79232) with fluid continually replaced at a rate of 6.15 mL/min. Proximal SI was isolated by making one cut distal to the stomach and a second cut ~2 cm proximal to the cecum. The proximal end of the SI was cannulated and tied to a P2/P10 Ultra Micro Pipet Tip (ThermoFisher, Cat# 02-707-438) connected using an L-shaped connector (Harvard Apparatus, Cat# 72-1407) and tubing to an upright 3mL syringe (Beckton Dickerson, Franklin Lakes, NJ; Cat# 309657). Fluid
level in the syringe was maintained at 1.3 cm above the proximal end of the bowel.

Proximal and distal bowel were at the same elevation. Bowel was pinned loosely on a secure piece of Sylgard 184 Silicone Elastomer (Dow Corning), illuminated to contrast with background, and filmed with a camera (Olympus, Center Valley, PA; PEN Mini E-PMI) connected to a dissecting microscope (Olympus SZ-PT SZ40). After an initial 20 minute video, 1 mM tetrodotoxin stock solution (Abcam, Cambridge, UK; Cat# ab120054) dissolved in sodium citrate buffer (40 mM citric acid monohydrate and 60 mM trisodium citrate dihydrate, pH 4.8) was diluted in Krebs-Ringer to a concentration of 1 µM. The resulting solution was oxygenated and pumped into the organ bath, and then another 20 minute video was recorded.

Analysis of bowel motility patterns

Videos were converted to .wmv format using Movie Maker and saved at 1x and 16x speeds. In-house MatLab (MathWorks, Natick, MA) scripts (https://github.com/christinawright100/BowelSegmentation) were used to threshold the movies, generate kymographs, and perform Fourier transform analysis. Peak frequency was determined by calculating the Fourier transform at each vertical slice of the kymograph, averaging these, and plotting the averages to determine the peak frequency. Investigators blinded to genotype and condition quantified neurally-mediated contractions in kymographs and recorded if contractions were rhythmic or non-rhythmic. To quantify bowel width and tortuosity, 10 random frames were saved from each video and analyzed in ImageJ. For each frame, we measured 5 random widths (randomly...
generated using a MatLab script) along the proximal bowel (50 widths per bowel). For tortuosity, total bowel length was divided by straight-line distance.

Quantitative PCR (qPCR) Analysis

Quantitative real time-polymerase chain reaction (qRT-PCR) was performed using previously described primers which we validated (Supplementary Table 3; Supplementary Figure 4) and SSoFast Evagreen (Bio-Rad, Hercules, CA; Cat# 172-5211). Fold change relative to control was computed using the ΔΔCt method with normalization to Gapdh mRNA levels.

RNA sequencing

RNA was extracted as described above. cDNA libraries were generated from samples with RIN > 8.0 using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio, Kusatsu, Japan; Cat# 634411) designed to work with very low input RNA. Libraries were sequenced on a HiSeq2500 to a read depth of 17-26 million paired-end reads per sample. Reads were mapped to the genome using the STAR RNA-seq aligner (61), normalized using Pipeline of RNA-seq Transformations (PORT) (62) and analyzed in R (The R Foundation, Vienna, Austria) using Limma's Voom method (63, 64). Datasets will be made available on the GEO database pending publication of this paper.

Statistics

We used Prism 7.03 (GraphPad Software, San Diego, CA) and SigmaPlot 11.0 (Systat Software, Chicago, IL) for statistical analysis. A two-sided Student's t-test or Mann-
Whitney Rank Sum Test (MWRST) was used when comparing two groups. When comparing multiple groups, a one-way ANOVA with post hoc multiple comparisons tests (Tukey) was used unless assumptions were not met, in which case we used a Kruskal-Wallis test with Dunn's post hoc multiple comparisons test. When comparing weight across multiple time points and groups, a two-way repeated measures ANOVA was used with post hoc multiple comparisons tests (Tukey). A cutoff of p < 0.05 was considered significant. For RNA-seq analyses, a false discovery rate-adjusted p-value (i.e. q-value) of 0.10 was considered significant. Data represent mean ± standard error of the mean (SEM). Investigators were blinded to condition for all quantifications.

Study approval

All animal experiments were approved by the Children's Hospital of Philadelphia IACUC.
**Author contributions**

C.M.W. and R.O.H. designed experiments, analyzed results, and wrote the manuscript. C.M.W., J.P.G., H.L.M., D.R.K., S.S., and B.A.M. performed experiments. All authors edited the manuscript.

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Figure 1: **Dlx1/2-/- and Dlx2-/-** mice have obvious abdominal distention at P0 due to bowel air accumulation, while **Dlx1-/-** mice exhibit poor weight gain but normal SI transit. (A-D) **Dlx1/2-/-** (A-B) and **Dlx2-/-** (C-D) mice exhibited dramatic bowel distention. Scale bar = 1 cm. (E) Bowel of **Dlx2-/-** mouse viewed under dissecting microscope. Note air-filled proximal small intestine (yellow arrowhead) with absence of air in distal small intestine (white arrowhead) and colon (green arrowhead). Scale bar = 5mm. (F) Control bowel for comparison. Scale bar = 5 mm. (G) Kaplan-Meier curve indicates most **Dlx1-/-** mice survived past 1 month of age (p=0.4665; Log-rank Mantel-Cox; n=9(+++), 16(+/-), 6(-/-)). (H-I) **Dlx1-/-** mice were small and weighed significantly less than their WT littermates at P15 (p = 0.0433), P20 (p<0.0001), P25 (p<0.0001), and
P30 (p<0.0001; n=9(+/+), 14(+/-), and 5(-/-); 2-way repeated measures ANOVA). We include only mice that lived to P35. (J) % FITC-dextran in distinct bowel regions 1 hour after oral FITC-dextran administration. SI1 to SI10 indicate sequential small intestine segments. (K) Weighted average of FITC bowel transit showed normal small intestine transit times in Dlx1-/- mice (p= 0.5182; one-way ANOVA, n=6(+/+), 4(+/-), and 4(-/-)). (L) Dlx1-/- mice had slight delays in expulsion of a glass bead from distal colon compared to WT controls (p = 0.0399, Student's t-test, n=5(+/+) and n=4(-/-)). *p<0.05, ****p<0.0001.
Figure 2: Dlx1/2−/− mice have abnormal bowel motility.

(A) Percent FITC-Dextran in distinct bowel regions 3 hours after oral FITC administration. SI1 to SI6 indicate sequential small intestine segments. (B) Weighted average of FITC bowel transit, represented by geometric mean, shows significant delays in transit for P0 Dlx1/2−/− mice, but not for heterozygous Dlx1/2+/− mice (p=0.0188; Kruskall-Wallis with Dunn's multiple comparisons test; n = 15 (+/+), 33 (+/−), 6 (−/−)).
(C) A subset of heterozygotes spontaneously accumulated bowel air, but transit in
Dlx1/2/- mice was still slower than in Dlx1/2+/+ mice. (p=0.0228; Student's t-test; n=7 (+/−), n=6 (-/-)). (D-G) Representative kymographs depicting bowel width (color axis) over time (y-axis) at specific distances along the small intestine (x-axis) in control and Dlx1/2/- bowels in the absence (D-E) or presence (F-G) of tetrodotoxin. White arrows indicate contraction complexes in control mice, but similar contraction complexes could not be identified in Dlx1/2/- bowel; white arrowheads indicate artifacts such as air bubbles on the surface of bowel. (H) Dlx1/2/- mice had fewer low-frequency (L.F.) contraction complexes than controls (p=0.0055; Kruskal-Wallis with Dunn’s multiple comparisons test; n=10 (ctrl), n=9 (-/-)). (I) Low-frequency contraction complexes generally occurred in rhythmic patterns in controls but not in mutants. (J) Representative Fourier plot from control mouse, with black arrow highlighting peak contraction frequency for TTX-insensitive contractions; note that contraction frequency for neurally-mediated contractions is too low to be reliably identified on the Fourier graph. (K) High-frequency (H.F.) TTX-insensitive contractions occurred at normal rates in Dlx1/2/- mice (p=0.181; one-way ANOVA; n=10 (ctrl), n=9 (-/-)). (L-M) In controls, average bowel width decreased significantly with addition of TTX (L; p=0.0043; one-way ANOVA with Tukey's multiple comparisons test), while tortuosity increased with TTX (M; p=0.0087; Kruskal-Wallis with Dunn's multiple comparisons test). In mutants, bowel width did not significantly change with TTX addition (L; p=0.1423), but tortuosity increased with TTX (M; p=0.0276). *p<0.05; **p<0.01; ***p<0.001. Error bars represent S.E.M.
Figure 3: Dlx1/2-/-, Dlx2-/-, and Ret+/-;Dlx1/2-/- mice have normal bowel colonization rates by ENC DC or minimal defects at E12.5. (A-B) Confocal Z-stacks of WT (A) and Dlx1/2-/- (B) E12.5 bowel stained with TuJ1 antibody that labels early and mature neurons (green). Maximum intensity projections are shown. White triangles mark distal-most TuJ1-positive neuron processes. Scale bar = 1 mm. (C-D) Percent hindgut colonized relative to total colon length (C; one-way ANOVA, n=7(+/+), n=13(+/-), n=6(-/-)) and colon length (D; one-way ANOVA, n=7(+/+), n=13(+/-), n=6(-/-)) was normal in Dlx1/2-/- mutants. (E-H) Representative WT (E) and Dlx2-/- (F) E12.5 bowel labeled with TuJ1 antibody. Dlx2-/- mice had a slight delay (66.5 ± 1.5% colonized (+/+) vs 47.4 ± 5.2% colonized (-/-)) in ENS migration of unknown functional significance (G; p =0.0499; Kruskal-Wallis test with Dunn's multiple comparisons test, n=4(+/+), n=15(+/-), n=7(-/-)) and normal colon length (H). (I-L) Representative Ret+/-
Dlx1/2+/+ (I) and Ret+/−;Dlx1/2−/− (J) E12.5 bowel labeled with TuJ1 antibody. Dlx1/2−/− mice on a Ret heterozygous background had normal extent of bowel colonization (I; one-way ANOVA, n=3 per genotype) and normal colon length (L; one-way ANOVA, n=3 per genotype). Scale bar = 1 cm. *p<0.05.
Figure 4: *Dlx1/2*−/− mice have normal density of neurons, SOX10+ cells, and glia at P0. (A-J) Representative images of *Dlx1/2*+/+ and *Dlx1/2*−/− bowel immunostained for HuC/D (magenta) and TuJ1 (green). MP=myenteric plexus, SP=submucosal plexus. (K-O) Quantification of neurons in (A-J) shows no difference between neuron number in control or mutant mice (Student's t-test; n=3-4 per condition). (P-S) Representative images of *Dlx1/2*+/+ and *Dlx1/2*−/− bowel immunostained for S100β (magenta) and
SOX10 (green). (T-W) *Dlx1/2-/-* mice had normal numbers of SOX10+ cells at P0 (Student's t-test; n=3-4 per condition; T-U) and normal numbers of SOX10+S100β+ glia at P0 (Student's t-test; n=3-4 per condition; V-W). Scale bar = 100 μm.
Figure 5: *Dlx1-/-* adults have normal neuron and glial numbers. (A-X) Representative images and quantifications of adult *Dlx1-/-* mouse proximal SI, distal SI, mid-colon, and distal colon immunostained using HuC/D (magenta) and TuJ1 (green) antibodies. Myenteric plexus (A-H) and submucosal plexus (M-T) are shown. Neuron density was normal in *Dlx1-/-* mice for all bowel regions analyzed (I-L, U-X; Student's t-test; n≥3 per
condition). (Y,Z) Adult Dlx1-/- distal SI was immunostained using an antibody against SOX10 which exclusively labels glia in adults (red). (A') Quantitative analysis of glial density shows no difference in glial cell density in distal small intestine of Dlx1-/- mice or WT mice (Student's t-test; n=3 per condition). Scale bar = 100 μm.
Figure 6: Dlx1/2-/- P0 and Dlx1-/- adult mice have normal ratios of neuron subtypes.

(A-L) Representative images of HuC/D (red), nNOS (blue), and ChAT-EGFP (green; A,G), HuC/D (magenta) and NADPH-diaphorase (green; B, H), HuC/D (magenta) and calretinin (green; C, I), HuC/D (magenta) and GABA (green; D, J), HuC/D (magenta) VIP (green; E-F, K-L) immunostaining in P0 control, P0 Dlx1/2-/-, and adult WT bowel. SP = submucosal plexus and MP = myenteric plexus. (M-R) We observed no difference in ratios of myenteric ChAT-EGFP or nNOS+ (M), myenteric NADPH+ (N), calretinin+ (O), GABA+ (P), VIP+ (Q) or submucosal VIP+ (R) neurons to total HuC/D+ neurons in Dlx1/2-/- mice compared to control mice. WT and Dlx1/2+/+ were grouped as “control”
and color coded $Dlx1/2^{+/+}$ (blue), $Dlx1/2^{+-}$ (green) (Student's t-test, n=3-6 per condition). (S-T; V-W; Y-Z, B'-C') Representative images of HuC/D (magenta) and nNOS (green; S-T), HuC/D (magenta) and GABA (green; V-W), HuC/D (magenta) and VIP (green; Y-Z), and HuC/D (magenta) and somatostatin (green; B'-C') immunostaining in adult control and $Dlx1/-$ ENS. SP = submucosal plexus and MP = myenteric plexus. (U, X, A', D') We observed no difference in myenteric nNOS+ (U) or GABA+ (X) neurons, and no difference in submucosal VIP+ (A') or somatostatin+ (D') neurons in $Dlx1/-$ ENS (Student's t-test, n=3 per condition). Arrowheads indicate examples of neurons that were counted. For GABA-ergic neurons in $Dlx1/-$ adults, white arrowheads indicate brightly-positive GABA-ergic neurons, while yellow arrowheads indicate dimly-positive GABA-ergic neurons. Scale bar = 100 μm and applies to all images in the set.
Figure 7: Vip levels are reduced in the developing ENS of Dlx1/2-/- mice at E14.5 and P0. (A) Bland-Altman plot of differentially expressed genes in Dlx1/2-/- versus WT E14.5 FACS-sorted small bowel ENCDC after RNA-seq. (B) Heatmap of differentially-expressed genes shows 20 dysregulated genes in Dlx1/2-/- ENS at E14.5, in addition to Dlx1 and Dlx2 which were substantially decreased in mutant mice. (C-D) Quantitative RT-PCR was performed for Vip (C) and Penk (D) mRNA on independent samples to
validate expression patterns for these neurotransmitters in E14.5 mouse ENS (Vip, p=0.0042, Student’s t-test, n=5(+/+) and n=4(-/-); Penk, p=0.0634, Student’s t-test, n=5(+/+) and n=4(-/-)). (E) Bland-Altman plot of differentially expressed genes in Dlx1/2-/- versus WT P0 FACS-sorted ENCDC after RNA-seq. (F) Heatmap of differentially-expressed genes shows 5 dysregulated genes in Dlx1/2-/- ENS at P0, in addition to Dlx1 and Dlx2. **p<0.001
Figure 8: Vip-lineage TdTome+ neuron numbers are decreased in Dlx1/2-/

Myenteric and submucosal plexus.

(A-B) Myenteric plexus from Dlx1/2\textsuperscript{wt/wt or +/-};VIP-IRESCre\textsuperscript{Cre/+/};R26R-TdTome+ control (A) and Dlx1/2-/-;VIP-IRESCre\textsuperscript{Cre/+/};R26R-TdTome+ mouse (B) stained with ANNA-1 antibody labeling HuC/D (green) reveals fewer TdTome+ cells in mice lacking Dlx1/2. (C-D) Quantification of (A-B) shows a reduced proportion of TdTome+ neurons in Dlx1/2-/- myenteric plexus (C; p=0.047, n=6(ctrl) and n=5(-/-)) and reduced overall TdTome+ neuron density (D; p=0.0017, n=6(ctrl) and n=5(-/-)). (E) Total MP neuron density was unchanged in Dlx1/2 mutant mice (E; p=0.26, n=6(ctrl) and n=5(-/-)).

(F-G) Dlx1/2-/-;VIP-IRESCre\textsuperscript{Cre/+/};R26R-TdTome+ mutants also have fewer TdTome+ (red) ANNA-1+ cells in their submucosal plexus. (H-I) Quantification of (F-G) showing decreased proportion of TdTome+ neurons relative to total (H; p=0.0098, n=5(ctrl) and n=4(-/-)) and decreased TdTome+ neuron density (I; p=0.0027, n=5(ctrl) and n=4(-/-)). (J) Total SP neuron density was unchanged in Dlx1/2-/- mice.

Scale bar = 100 µM.
Figure 9: *Dlx1/2-* mice have apparently normal large nerve fiber bundles but decreased CSM neurite density on some strain backgrounds. (A-H) Myenteric plexus labeled with TuJ1 (green; A,E), TdTomato (red; B,F), HuC/D (blue; C,G). All large nerve fiber bundles (white arrowheads) were TdTomato+. (I-J) Myenteric plexus nerve fiber bundle width (white line, I) was similar in *Dlx1/2-* and control mice (J; p=0.4565, Student's t-test, n=4 per genotype). (K-P) Circular muscle neurites labeled with TuJ1 (green; K,N) and TdTomato (magenta; L,O) from P0 bowel. Some neurites were TdTomato and TuJ1+ (white arrowheads), while others were TuJ1+ alone (white arrows). (Q) Density of total TuJ1+ neurites was decreased in *Dlx1/2-;Vip-IRES-Cre;TdTomato*
P0 bowel (p=0.0077, Student's t-test, n=4(ctrl) and n=3(-/-)). (R) Ratio of TdTomato+ neurites to TuJ1+ neurons was unchanged (p=0.5587, Student's t-test, n=4(ctrl) and n=3(-/-)). (S) Density of TdTomato+ neurites was not statistically different for control and *Dlx1/2/-* mice (p=0.1006, Student's t-test, n=4(ctrl) and n=3(-/-)). (T) Density of total TuJ1+ neurites was normal for *Dlx1/2/-* mice on a CD1 background (p=0.3193, Student's t-test, n=4(ctrl) and n=4(-/-)). Scale bar=100 µM for D-H and K-P, and 50 µM for I.
Figure 10: VIP is reduced in Dlx1/2−/− nerve fibers and cell bodies

(A-H) High-resolution slices from confocal Z-stacks showing TuJ1 (red), HuC/D (blue), and VIP (green). (I,J) Example mask constructed by thresholding on the TuJ1 channel and subtracting thresholded HuC/D. (K) Average VIP intensity within masked regions was significantly reduced in Dlx1/2−/− mutant mice compared to control, suggesting reduced VIP expression in nerve fibers (p=0.0039, Student's t-test, n=3(+/+), n=3(-/-)).

(L-N) Total area of pixels with high VIP (defined arbitrarily as VIP intensity > 0.2 A.U.; L) was divided by total neurite area (M) to determine the % of nerve fiber bundles with high VIP expression, an approximation for density of VIP varicosities. This value was 51
significantly reduced in Dlx1/2-/ nerve fibers (p=0.0070, Student's t-test, n=3(+/+), n=3(-/-); N). (O,P) Example mask constructed by thresholding on the HuC/D channel. (Q)

Average VIP intensity within soma was not significantly decreased in Dlx1/2-/ mice relative to controls (p=0.100, MWRST, n=3(+/+) and n=3(-/-)). (R-T) Percent soma area with high VIP expression was significantly decreased in Dlx1/2-/ mice (p=0.0033, Student's t-test, n=3(+/+) and n=3(-/-)).
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