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Retinaldehyde dehydrogenase enzymes regulate colon enteric nervous system structure and function

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ABSTRACT

The enteric nervous system (ENS) forms from the neural crest-derived precursors that colonize the bowel before differentiating into a network of neurons and glia that control intestinal function. Retinoids are essential for normal ENS development, but the role of retinoic acid (RA) metabolism in development remains incompletely understood. Because RA is produced locally in the tissues where it acts by stimulating RAR and RXR receptors, RA signaling during development is absolutely dependent on the rate of RA synthesis and degradation. RA is produced by three different enzymes called retinaldehyde dehydrogenases (RALDH1, RALDH2 and RALDH3) that are all expressed in the developing bowel. To determine the relative importance of these enzymes for ENS development, we analyzed whole mount preparations of adult (8–12-week old) myenteric and submucosal plexus stained with NADPH diaphorase (neurons and neurites), anti-TuJ1 (neurons and neurites), anti-HuC/HuD (neurons), and anti-S100β (glia) in an allelic series of mice with mutations in *Raldh*1, *Raldh*2, and *Raldh*3. We found that *Raldh*1$^{-/-}$, *Raldh*2$^{+/-}$, *Raldh*3$^{+/-}$ (R1^{KO}R2^{Het}R3^{Het}) mutant mice had a reduced colon myenteric neuron density, reduced colon myenteric neuron to glia ratio, reduced colon submucosal neuron density, and increased colon myenteric fibers per neuron when compared to the wild type (WT; *Raldh*1^{WT}, *Raldh*2^{WT}, *Raldh*3^{WT}) mice. These defects are unlikely to be due to defective ENS precursor migration since R1^{KO}R2^{Het}R3^{KO} mice had increased enteric neuron progenitor migration into the distal colon compared to WT during development. RALDH mutant mice also have reduced contractility in the colon compared to WT mice. These data suggest that RALDH1, RALDH2 and RALDH3 each contribute to ENS development and function.

Introduction

The enteric nervous system (ENS) controls most aspects of the intestinal function (Wood et al., 1999). To perform this task, the ENS contains an integrated network of neurons and glia within the bowel wall estimated to contain at least 20 different types of neurons (Furness, 2006). ENS abnormalities can cause intestinal motility disorders leading to abdominal pain, constipation, diarrhea, vomiting, malnutrition and death (Faure, 2013; Obermayr et al., 2013). Elucidation of the molecular mechanisms controlling ENS development may therefore lead to new ways to prevent or treat the intestinal motility disorders.

The active vitamin A metabolite retinoic acid (RA) is known to be essential for normal ENS development. Studies *in vitro* demonstrated that blocking RAR signaling or depriving cells of RA reduced ENS precursor proliferation, reduced neuronal differentiation, and increased neurite length (Sato and Heuckeroth, 2008). Furthermore, retinoid deficiency *in vivo* from E7.5 to E14.5 impaired ENS precursor colonization of the bowel leading to distal bowel aganglionosis resembling human Hirschsprung disease (Fu et al., 2010). RA affects development by binding to RAR and RXR receptors that in turn regulate transcription. Because RA is produced locally in tissues from retinol, RA signaling depends on the rate of RA synthesis and degradation (Duester, 2008). RA is synthesized by three retinaldehyde dehydrogenase enzymes expressed in the developing bowel (RALDH1, RALDH2 and RALDH3) (Sato and Heuckeroth, 2008). Previous studies demonstrated that *Raldh*2 homozygous null embryos die by E10.5 due to severe early heart defects, unless given supplemental RA from E7.5

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to E9.5 that allows survival until E14.5 (Kumar et al., 2012; Niederreither et al., 2001, 2003). However, even with early RA supplementation that allows longer survival, *Raldh*2 null embryos fail to form an enteric nervous system (Niederreither et al., 2002), demonstrating that *Raldh*2 function after E9.5 is absolutely essential for ENS development. Mice homozygous for null *Raldh*1 mutations are fertile and survive without any apparent developmental anomalies (Fan et al., 2003). Mice homozygous for null *Raldh*3 mutations die just after birth of respiratory distress secondary to choanal atresia (Dupé et al., 2003), but the role of RALDH1 and RALDH3 in ENS development has not been investigated. In this study, our goal was to determine the relative importance for ENS development of each of the three RALDH enzymes expressed in the developing bowel. We found that compound *Raldh* mutant mice had reduced colon myenteric and submucosal neuron density, reduced colon glial cell density, increased colon myenteric neuronal fiber density per neuron, and we demonstrated defects in the peristaltic response of *Raldh* mutant mice to mucosal stimulation. From these data, we conclude that RALDH1, RALDH2 and RALDH3 each influence ENS development and function.

Materials and methods

Animals

All animal experiments were in compliance with institutional animal protocols approved by the Washington University Animal Studies Committee. *Raldh*$1^{-/-}$ (Fan et al., 2003), *Raldh*$2^{+/-}$ (Mic et al., 2002), and *Raldh*$3^{+/-}$ mice (Molotkov et al., 2006) on a mixed 129SvJ, Black Swiss, and C57Bl/6 background were bred to produce *Raldh*1^{WT}, *Raldh*2^{WT}, *Raldh*3^{WT} and *Raldh*$1^{-/-}$, *Raldh*$2^{+/-}$, *Raldh*$3^{+/-}$ animals, as well as several intermediate genotypes. Mice were genotyped using the PCR primers listed in Table 1.

Quantitative ENS analysis

Mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. Myenteric and submucosal plexus samples were obtained as described previously (Viader et al., 2011). In the adult mice, sequential 2 cm long samples of myenteric plexus taken from the duodenum (starting at the junction with the pylorus) and distal colon were double-labeled with either biotinylated-anti-HuC/HuD (mouse; polyclonal; 1:250; Invitrogen A21272) and anti-S100β (rabbit; polyclonal; 1:500; Dako), or NADPH diaphorase (NADPH-d) (Neuhuber et al., 1994) and anti-TuJ1 (rabbit; polyclonal; 1:10,000; Covance). Submucosal plexus samples (2 cm long) from the proximal small intestine (SI) and distal colon were labeled with acetylcholinesterase (AChE) as described previously (Enomoto et al., 1998), a method that stains all mouse submucosal neurons that are labeled by TuJ1 (Wang et al., 2010). In E13.5 mice, whole gut samples were labeled with anti-TuJ1 (1:10,000; Covance). Briefly, bowel was incubated in TBST (100 mM Tris, 150 mM NaCl, 0.5% Triton X-100) for 15 min at 37 °C, blocked in 5% normal donkey serum (NDS)/TBST for 30 min at 37 °C and then incubated in primary antibody in TBST/5% NDS overnight at 4 °C. Samples to be stained with biotinylated-anti-HuC/HuD were treated with 3% H_2O_2 (in PBS) (20 min, 25 °C), incubated in PBST (1× PBS, 1% Triton X-100, 30 min, 25 °C), and then blocked using the Vector SA-Biotin Blocking Kit, per manufacturer's protocol. Samples were subsequently blocked in 10% NDS/PBST (45 min, 25 °C) before incubating in biotinylated-anti-HuC/HuD antibody (in 10% NDS/PBST) overnight at 4 °C.

Table 1
PCR primers used for genotyping.

Primer	Sequence
RALDH1 WT F1	TAAAGACCTGGATAAGGCCATCACTGTGTC
RALDH1 WT R1	CCGCCGAGGCACCAACACATTCTCTAACGTG
RALDH1 mutant (Neo3′)	CGCCAAGCTCTTCAGCAATATCACGGGTAG
RALDH1 mutant (Neo5′)	TGCTCCTGCCGAGAAAGTATCCATCATGGC
RALDH2 WT F1	GAAGCAGACAAGGTGTGTATTGCTTAGAAG
RALDH2 WT R1	GCTTGCACTGCCTTGTCTATATCCACCTGT
RALDH2 Mutant F1	GCCTGACCTATTGCATCTCCCG
RALDH2 Mutant R1	GCCATGTAGTGTATTGACCGATTCC
RALDH3 F2	CAGCCGTCACACAGTTATAGAGGTCTCTGG
RALDH3 WT R2	TCGAGCGCAGCAGCCAGCTTCAGTGCTTTG
RALDH3 Mutant R2	GTTGCAAAACACGTAGGTGCATGCAGGAGA

Quantification of neuron or glial cell density was done by counting all the cells present within the borders of a 0.5×0.5 mm^2 grid (20× objective). Neuronal fiber density was analyzed in the circular and longitudinal muscle layers that contain the myenteric plexus by counting "fibers" or "bundles" of fibers that cross perpendicular axes of the same counting grid as described previously (Wang et al., 2010). Supplemental Fig. 1 shows how "fibers" and "fiber bundles" were counted. An attempt was made to stretch segments evenly for all the samples. Counting was done without the knowledge of the genotype. Twenty randomly selected fields were counted for each region and staining method. Data are presented per square millimeter. Neuron-to-glia ratio was determined by dividing the number of neurons by the number of glia within a single field. Quantification of extent of colon colonization by ENS precursors in E13.5 mice was accomplished by stitching 4× images using Fiji/ImageJ (Preibisch et al., 2009) and measuring colon length from the tip of the cecum to the anus and the length of the segment colonized by ENS precursors from the tip of the cecum to the most distal TuJ1$^+$ neuronal cell body or neurite.

Light microscopy and quantification

Samples were counted on an Olympus BX60 or Zeiss Axioskop microscope. Images were obtained with an Olympus BX60 or Axioplan2 microscope equipped with an Axiocam digital camera using AxioVision imaging software (Zeiss, Germany). Fiji/ImageJ (1.47d) was used to uniformly adjust contrast and brightness so that digital images appear as when observed directly through the microscope.

Functional motility studies

Colonic segments were removed and cleared of luminal contents by gentle flushing with Krebs bicarbonate buffer. Colonic segments of about 5 cm length were opened along the mesenteric attachment to make a flat-sheet preparation and pinned mucosal side up in a 3-compartment organ chamber as described in detail previously (Grider, 2003; Wang et al., 2010). 1 mL of a Krebs-bicarbonate medium was added to each compartment (mM: 118 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 NaH_2CO_3, 11 glucose). The mucosa of the central compartment was stimulated by stroking with a fine brush (2–8 strokes, 1 stroke/s). Ascending contraction of circular muscle was measured in the orad peripheral compartment and descending relaxation was measured in the caudad peripheral compartment using force–displacement transducers attached to the muscle layers. Results are expressed as grams force above or below baseline tone.

Statistical analysis

All values are expressed as mean ± SEM. Averages for each animal were used for statistical analyses. p values were