Published in final edited form as:

J Pediatr Surg. 2014 August ; 49(8): 1242–1251. doi:10.1016/j.jpedsurg.2014.01.060.

Intestinal Dysbiosis and Bacterial Enteroinvasion in a Murine Model of Hirschsprung's Disease

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Abstract

Background/Purpose—Hirschsprung's disease (HSCR), characterized by the absence of ganglia in the distal colon, results in functional obstruction. Despite surgical resection of the aganglionic segment, around 40% of patients suffer recurrent life threatening Hirschsprung's-associated enterocolitis (HAEC). The aim of this study was to investigate whether gut microbiota and intestinal immunity changes contribute to the HAEC risk in a HSCR model.

Methods—Mice with neural crest conditional deletion of Endothelin receptor B (*EdnrB*) and their littermate controls were used (*EdnrB*-null and *EdnrB*-het). Bacterial DNA was prepared from cecal contents of P16–18 and P21–24 animals and pyrosequencing employed for microbiome analysis. Ileal tissue was isolated and secretory phospholipase A₂ (sPLA₂) expression and activity

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COMPETING INTERESTS

None

PROVENANCE AND PEER REVIEW

Not commissioned; externally peer reviewed.

CONTRIBUTORSHIP

AG was responsible for conception and design. AG, JFP, AJBA, CSE, AFH, SED were responsible for acquisition of data. AG, JFP, AJBA, AFH, SED, MLE, KAK were responsible for analysis & interpretation of data. JFP, AJBA, CSE and AG were responsible for drafting of the manuscript. AJBA, JFP and AG were responsible for critical revision of the manuscript.

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determined. Enteroinvasion of *E. coli* into ileal explants was measured using an *ex vivo* organ culture system.

Results—*EdnrB*-het and *EdnrB*-nulls displayed similar flora, sPLA₂ expression and activity at P16–18. However, by P21–24, *EdnrB*-hets demonstrated increased *Lactobacillus* and decreased *Bacteroides* and *Clostridium*, while *EdnrB*-nulls exhibited reciprocal changes. *EdnrB*-nulls also showed reduced sPLA₂ expression and luminal activity at this stage. Functionally, *EdnrB*-nulls were more susceptible to enteroinvasion with *E. coli ex vivo* and released less sPLA₂ than *EdnrB*-hets.

Conclusions—Initially, *EdnrB*-het and *EdnrB*-nulls contain similar cecal flora but then undergo reciprocal changes. *EdnrB*-nulls display dysbiosis, demonstrate impaired mucosal defense, decreased luminal sPLA₂ and increased enteroinvasion of *E. coli* just prior to robust colonic inflammation and death. These findings suggest a role for the intestinal microbiome in the development of HAEC.

Keywords

Hirschsprung's Disease; Hirschsprung's-associated enterocolitis; Microbiome; Secretory Phospholipase A2; Dysbiosis; Mucosal Immunity

INTRODUCTION

Hirschsprung's disease (HSCR) is characterized by congenital segmental absence of the enteric nervous system (ENS) in the distal gut due to a failure of neural crest cell (NCC) migration during embryonic development [1,2]. HSCR results in intestinal obstruction that is typically treated by surgical resection of the aganglionic bowel and a "pull-through" of ganglionated bowel. Unfortunately, up to 40% of patients continue to suffer recurrent Hirschsprung's-associated enterocolitis (HAEC)[3,4], suggesting that susceptibility is not solely dependent upon the presence of the obstruction. The pathogenesis and etiology of HAEC have not been clearly determined although alterations in host innate immunity, intestinal barrier function and gut microflora have been proposed to contribute to HAEC susceptibility [3,4]. Clinical and animal investigations of HAEC have attempted to identify specific organisms that promote enterocolitis development [5–7]. A single, causative organism has not been isolated, but rather a handful of potential contributors, including *Clostridium difficile*, *Escherichia coli*, and viruses have been suggested. Medical management of HAEC is non-specific and consists of bowel rest, rectal irrigations to decrease stool burden and systemic antibiotic therapy with metronidazole [4], suggesting that alterations in the gut microbiota composition may underscore HAEC susceptibility.

Determination of gut microbiome composition has been advanced by the development of culture-independent analysis. Traditional, culture-dependent methods are limited to strains that can be grown using current laboratory protocols [8]. Culture-independent techniques can examine all bacterial strains present in the gut by sequencing the genes that encode bacterial 16S ribosomal RNA [9]. These techniques have been employed to investigate the possible role of the microbiome in a number of pathological processes, including obesity, diabetes mellitus, inflammatory bowel disease, and irritable bowel syndrome [10–13]. Many

of these investigations demonstrate a shift in the microbiome composition of patients compared to healthy individuals, with relative over- or under-representation of specific taxa or species. For instance, greater levels of certain *Lactobacillus* species have been shown to be protective due to anti-inflammatory effects and to their competitive exclusion of some pro-inflammatory *Bacteroides* species [14,15].

The microbiome population of the intestinal lumen is regulated in-part by mucosal secretions [16]. Paneth cells, which reside at the base of the intestinal crypts, directly sense the microbiota and maintain homeostasis through secretion of antimicrobial molecules, including secretory phospholipase A₂ (sPLA₂), lysozyme, and defensins/cryptidins [16–19]. sPLA₂ functions to disrupt bacterial cell membranes of both gram-positive and gram-negative bacteria [20]. Additionally, loss of Paneth cell antimicrobial products results in dysbiosis of the intestinal microbiome that can lead to increased tissue inflammation and colitis [21–23]. To date, an examination of host small intestinal mucosal barrier dysfunctions that affect pathogen susceptibility, such as the loss of antimicrobial protein production and secretion by Paneth cells, has not been undertaken within the context of HAEC.

Multiple genetic defects are associated with HSCR, most commonly mutations of the receptor tyrosine kinase gene, *Ret*, and *endothelin receptor B* (*EdnrB*) [1,2]. In addition to its role in ENS formation, *Ret* is required for development of Peyer's patches (PP), the primary inductive site for gastrointestinal adaptive immune function, suggesting a potential developmental link between the ENS and mucosal immunity [24]. However, *Ret* knockout mice are unsuitable as a HAEC model since they exhibit a severe phenotype that includes renal agenesis and die shortly after birth [25]. In order to investigate the etiology of HAEC, we utilized mice with a NCC deletion of *EdnrB* (*EdnrB*^{flex3/flex3}, hence forth called *EdnrB*-null) [26]. *EdnrB*-null mice closely recapitulate the phenotypic traits observed in humans, demonstrating colonic aganglionosis. Additionally, *EdnrB*-null mice develop enterocolitis by post-natal day (P) 24–26 that is lethal around P28 and are ideally suited for the study of HAEC [26].

We hypothesize that changes in the microbiota as well as defects in gastrointestinal mucosal immune defense precede HAEC onset, alterations that may explain susceptibility. In this study we investigated whether changes in the microbiota were associated with HAEC development in our HSCR model by examining cecal content samples from *EdnrB*-het and *EdnrB*-null animals at two time points, P16–P18 and P21–P24, prior to robust colitis and mortality in *EdnrB*-null animals. We used *Ex Vivo* Intestinal Segment Culture (EVISC) [27] to determine if *EdnrB*-nulls have increased susceptibility to bacterial enteroinvasion compared to *EdnrB*-het animals. Finally, we determined if susceptibility to enteroinvasion and development of HAEC may in part be explained by alterations in sPLA₂ levels or activity in the ileum.

MATERIALS AND METHODS

Animals

All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee. We utilized a mouse model with NCC deletion of endothelin receptor B (*EdnrB^{flex3/flex3}*) [26]. Briefly, mating *TgWnt1-Cre/+; EdnrB^{flex3/+}* mice with *Rosa26^{YFPStop/YFPStop}; EdnrB^{flex3/flex3}* or *Rosa^{26tdTomato Stop/tdTomato Stop}; EdnrB^{flex3/flex3}* mice resulted in either heterozygous (*EdnrB^{flex3/+}*) or homozygous deletion of *EdnrB* (*EdnrB^{flex3/flex3}*), (defined throughout the manuscript as *EdnrB*-het and *EdnrB*-null, respectively). In addition, these mice express either yellow fluorescent protein (YFP) or tdTomato protein in their NCC. *EdnrB*-null animals display distal colonic aganglionosis of 5–10 mm in length [28] that can be identified by the absence of fluorescent NCC in their distal colon [26]. Mice were housed in a non-sterile environment and were allowed *ad libitum* access to food and water. To minimize cohabitation and litter effects on the microbiota studies [29], animals from multiple litters and cages were used over a 4 month period. For microbiome analysis, mice were divided into 4 groups (n=9–15 per group, per time point): *EdnrB*-het early (postnatal day 16–18, P16–P18), *EdnrB*-null early (P16–P18), *EdnrB*-het late (P21–P24) and *EdnrB*-null late (P21–P24). *EdnrB*-het mice are available from Jackson Laboratories (Stock Number: 009063).

Histology

Segments of colon and ileum were harvested from *EdnrB*-het and -null animals at P16–18, P21–24, and P26 (n=4 animals per group, per time point). Fresh specimens were fixed in 10% neutral buffered formalin for 24 hours and submitted for further processing into paraffin blocks. Paraffin-embedded small intestine and colon specimens were sectioned at a thickness of 5 microns, deparaffinized in xylene and either stained with routine hematoxylin & eosin (H&E) or Periodic Acid-Schiff reagent (PAS). The PAS staining protocol consisted of sections oxidized with 0.5% periodic acid for 7 minutes followed by a tap water rinse and subsequent immersion in Schiff reagent (Fisher Scientific, SS32–500) for 20 minutes. Finally, the tissue sections were washed in running warm tap water for development of magenta coloration and counterstained with hematoxylin for contrast.

DNA Extraction

Cecal content samples were homogenized and 200mg of each sample used to prepare DNA using the Qiagen DNA Stool Kit according to the manufacturers protocol (Qiagen, Valencia, CA). DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France).

Massively Parallel bTEFAP

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed using Gray28F 5'-TTTGATCCTGGCTCAG and Gray519r 5'-GTNTTACNGCGGCKGCTG, as previously described [8,9,30]. Initial generation of the sequencing library utilized a one-step PCR with 30 cycles, a mixture of Hot Start and HotStar high fidelity taq polymerases, and amplicons originating and extending from the 28F region for bacterial diversity. Tag-

encoded FLX amplicon pyrosequencing analyses utilized the Roche 454 FLX instrument with Titanium reagents. Procedures were performed at the Research and Testing Laboratory (Lubbock, TX) based upon RTL protocols (www.researchandtesting.com).

Bacterial Diversity Analysis

Sequencing data (average of 11589 sequences per sample, range 6716–18994) was analyzed by MR DNA (www.mrdnalab.com) after trimming low quality ends <Q25 and removing primers and tags, all failed sequence reads, sequences without 100% identity to expected barcode, sequences less than 200bp in length, sequences with degenerate/ambiguous bases, and sequences with homopolymer stretches > 6bp. Sequence collections were de-noised, de-multiplexed, and chimeras removed using USearch and UChime (Drive5.com). Sequence data was then clustered into Operational Taxonomic Units (OTUs) with 3% divergence using uClust. To determine the identity of bacteria in the sequence collection, sequences were de-noised, assembled into clusters and queried with BLAST+ against a database of high quality bacterial 16S rRNA sequences derived from GreenGenes (10-2011 version). Using a .NET and C# analysis pipeline, we compiled the resulting BLASTn outputs, validated them using taxonomic distance methods and data reduction analysis.

Taxonomic Determination

Based upon the BLAST+ derived sequence identity, bacteria were classified at the taxonomic levels using the following criteria. Sequences with identity scores to known or well characterized 16S rRNA sequences, greater than 95% were resolved to the genus level, 90–95% to the family level, 85–90% to the order level, 80–85% to the class level and 77–80% to the level of phyla. Here we report the analysis at the phylum and genera level; our other data are available upon request. After assessment, the percentage of each bacterial ID was individually analyzed for each sample providing the relative bacterial abundance within and among the individual samples. Evaluations presented at each taxonomic level, including percentage compilations, represent all sequences resolved to their primary identification or their closest relative, as has been previously described [31]. Organisms identified with sequence reads representing less than 1% of the total population (i.e. <10 sequence reads per 1000 total sequence reads) were excluded from the analysis. Hierarchical clusters of bacterial phylum, class, order, family, genus and species were obtained using Ward's minimum variance method. Trees and semi-partial R-squared values are presented and were produced using SAS version 9.2 (SAS Institute, Inc., Cary, NC). To assess the alpha diversity of the microbiota, the Rarefaction (OTU), Chao1, and Shannon-Weaver indices were calculated using the core pipeline of QIIME 6 [32]. Alteration of microbial communities among the 4 groups was investigated using principal coordinate analysis (PCoA) based on the phylogeny weighted Unifrac distance metric [33,34]. PCoA graphs were plotted using Past 2.17 (Oslo, Norway). ANOVA with Tukey-Kramer post hoc analysis was performed with xlstat (Addinsoft, NY). Dual dendrograms were constructed with NCSS 2007 (Kaysville UT).

Continuous fluorescent assay for sPLA₂ Activity

Fluorescent assay for sPLA₂ activity was performed as described previously [35], with modification of substrate preparation (n=12 per group, per time point)[19]. We utilized, a

Bis-BODipy FL specific probe, designed to fluoresce upon cleavage of the Sn₂ position of the phospholipid glyceraldehyde backbone. Upon reaching equilibrium, the fluorescent output was fit to a second-order polynomial equation and the initial rate of reaction was determined using a first-degree coefficient (expressed as FI/min/ μ L sample). Background activity was calculated from wells containing only substrate and buffer. This method, used previously in our laboratory, provides a high throughput analysis of sPLA₂ activity [19].

Bacterial Preparation for *Ex Vivo* Intestinal Segment Culture

Extraintestinal pathogenic *Escherichia coli*-Lux (ExPEC Strain 5011) containing ampicillin resistance were grown in lysogeny broth (LB) for 48 hours at 37°C under 5% CO₂, a surface sample was placed into fresh LB and grown for 24 hours at 37°C under 5% CO₂. Bacteria were centrifuged at 1780g for 11 minutes, the supernatant was removed, the pellet was re-suspended in 40ml LB before re-centrifugation and final re-suspension in 1 mL Dulbecco's phosphate buffered saline (DPBS) at 4°C as a bacteria stock solution. Bacterial stock solutions were diluted 1:100 and their concentration measured on a spectrophotometer (DU-640, Beckman, Urbana, IL) at 450nm wavelength. Bacterial concentrations were adjusted based on growth curves previously established in our laboratory.

Ex Vivo Intestinal Segment Culture (EVISC)

Enteroinvasion was assayed using EVISC (n=7–9 per group), as described previously [27]. Briefly, distal ileal segments, excluding Peyer's patches, were opened longitudinally along the mesentery. Dermabond tissue glue (Ethicon, Cornelia, GA) was applied to the surface of a tissue disc (6 mm internal diameter polystyrene) and the intestinal segments were attached apical (mucosal) side up. Once the glue set, the tissue disc was lowered into a cell culture insert (Cat: 3292, 3.0 μ M pore, 12 well format, BD bioscience, NJ). Cell culture inserts were placed into 12 well plates prefilled with 1 ml RPMI and Ampicillin (100 μ g/mL).

400 μ L of bacterial inoculum (1×10^8 Colony Forming Units (CFU)/mL) in RPMI+Ampicillin was placed in wells for 1 hour at 37°C. Following the bacterial challenge, the bacterial inoculum was collected, centrifuged at 14,000g for 2 minutes to pellet bacteria, and the supernatant was stored at –80°C for analysis of mucosal secretions. The wells were rinsed 3 times with 400 μ L of DPBS. Then 600 μ L RPMI containing Gentamicin (100 μ g/mL) was added to each well for 1 hour at 37°C to kill remaining bacteria in the well or adherent to the mucosal surface. RPMI+Gentamicin was then removed and the wash step repeated prior to the addition of 500 μ L of 0.1 % Triton-X in PBS to each well. The 12 well plates were placed on an orbital shaker and agitated (175rpm; New Brunswick Scientific Classic Series C1 Shaker) for 30 minutes at room temperature. Serial dilutions of cell lysates were made in DPBS and plated on LB containing ampicillin (100 μ g/mL) agar plates that were grown for 18 hours at 37°C. Enteroinvasion was assessed by determining colony-forming units (CFUs).

sPLA₂ Expression in Ileum Segments

To determine sPLA₂ expression in Paneth cell granules, we performed immunohistochemistry on segments of ileum as previously described [35]. Briefly, samples were fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, and processed in

a Tissue-Tek V.I.P processor. Samples were then embedded in paraffin, 5µm microtome sections cut and placed on adhesive coated slides (white Aminosilane, Newcomer Supply, Madison, WI). Antigen retrieval was performed by boiling slides in 10mM sodium citrate buffer (pH 6.0). Samples were incubated with primary antibody (1:2000, group II sPLA₂ (G-15) goat polyclonal IgG, sc-14468, Santa Cruz Biotechnology) overnight in 1% BSA-PBS at 4°C and then in the dark with secondary antibody (1/20,000, Alexa Fluor 594, donkey anti-goat IgG, 2mg/mL, A11058, Invitrogen, Grand Island, NY) for 30 minutes in 1% BSA-PBS at room temperature. DAPI (P36935, Invitrogen) was applied to image nuclei.

RESULTS

EdnrB-null animal survival and Histology

Previous studies of the naturally occurring *EdnrB* mutation (piebald lethal mouse strain) and the conventional *EdnrB* deletion have documented the development of enterocolitis prior to death and a mean survival time between 3–4 weeks of age [36,37]. In order to confirm the utility of the neural crest-conditional deletion *EdnrB*-null model in the study of HAEC, animals were followed until moribund and survival recorded (Figure 1A). Prior to sacrifice, these mice displayed ruffled hair coats, lethargy, shivering and anorexia. We observed that the majority of animals die between P21–33, with the median survival at 29 days and mean at 28.2 days. Of note, two animals died quite early, at P11–12, and two animals survived until P45–47. It is likely that both of these extremes of survival were related to access to nutrition.

Next, we harvested colonic tissues from *EdnrB*-het and -null animals at P21 and P26 to evaluate for inflammation (Figure 1B). In keeping with published results, we noted only the occasional presence of neutrophils in some of the P21 *EdnrB*-null colons, but found a robust inflammatory cell infiltrate present at P26 [37]. As with the survival data, it is likely that there is a spectrum of colonic inflammation between these two points.

Microbiome Taxonomic Composition

To determine the composition of the microbiota, we obtained cecal content samples from *EdnrB*-het and *EdnrB*-null mice at P16–18 (early) and P21–24 (late). The cecal region was chosen because there is an equivalent ENS within both of the genotypes [26]. Additionally, the *EdnrB*-null animals do not pass stool pellets per rectum and fecal material accumulates proximal to the aganglionic segment of the colon. Hierarchical clustering analysis of *EdnrB*-hets and *EdnrB*-nulls at both early and late time points (4 experimental groups) was performed at each taxonomic level from phylum through species. At the phylum level, the first order branching separated a group containing 45% *EdnrB*-null Late, 30% *EdnrB*-null Early and 25% *EdnrB*-het Early, from a group containing 46% *EdnrB*-het Late, 21% *EdnrB*-null Late, 18% *EdnrB*-het Early and 14% *EdnrB*-null Early (semi-partial R-squared=0.6119, Figure 2A). To further understand the phyla contributing to these groupings, the top three phyla found at the early time point were analyzed between groups (Figure 2B). The *EdnrB*-hets demonstrated decreased representation of *Bacteroidetes* and *Proteobacteria* over time, with increased *Firmicutes*. Of note, in the *EdnrB*-het Late animals, the second-most frequent

phylum was *Actinobacteria* (1.4%) and the third-most frequent was the candidate phylum TM7 (1.1%), both of which were represented at less than 1% in *EdnrB*-null Late animals.

Clustering analysis demonstrated similar separation of experimental groups at the lower taxonomic levels (class, order, family, genus, species), with *EdnrB*-het Late and *EdnrB*-null Late contributing the largest percentages to each of the first-order branches (Figure 2C). Because genus was the lowest taxonomic level with reliable separation between the experimental groups (semi partial R-squared of 0.4886), further analyses were performed at this taxonomic level.

At the genus level, the first order branching separated a group containing 45% *EdnrB*-null Late, 27% *EdnrB*-het Early and 27% *EdnrB*-het Late, from a group containing 79% *EdnrB*-het Late, 14% *EdnrB*-het Early and 7% *EdnrB*-null Early (Figure 2C). The top three genera present within all four experimental groups were compared by ANOVA (Figure 2D). *Lactobacillus*, many species of which are considered to be protective to the host mucosa through direct anti-inflammatory effects [14], were relatively similar between *EdnrB*-het and *EdnrB*-null animals at the early time point. However, more dramatic differences were evident between the *EdnrB*-het and *EdnrB*-null animals at the late time point, where *Lactobacillus* represented around 80% of the microbiome in *EdnrB*-hets compared with only 15% in *EdnrB*-nulls ($p < 0.05$). Of note, *Lactobacillus* increased over time in the *EdnrB*-hets while decreasing in the *EdnrB*-nulls ($p < 0.05$). In addition, at the late time point, *EdnrB*-nulls demonstrated reciprocal changes in two other bacterial genera, *Bacteroides* and *Clostridium*, both of which are commonly associated with mucosal inflammation [15]. The percentages of *Bacteroides* and *Clostridium* in *EdnrB*-nulls were significantly elevated compared with *EdnrB*-het samples at the late time point (*Bacteroides*: $0.2 \pm 3.5\%$ *EdnrB*-Het Late vs. $15.9 \pm 3.1\%$ *EdnrB*-Null Late, *Clostridium*: $2.8 \pm 2.6\%$ *EdnrB*-Het Late vs. $12.7 \pm 2.3\%$ *EdnrB*-Null Late, $p < 0.05$), suggesting a relative over-representation of these two genera prior to the development of robust inflammation and mortality. Detailed statistical differences in individual genera across each experimental group are displayed in Table 1.

Finally, principal coordinate analysis was performed to detail the contributions of the most abundant bacterial populations within each of the samples (Figure 3). This analysis demonstrated that *EdnrB*-het and -null Early samples were similar in composition, displaying overlap of the 95% confidence interval ellipses. However, at the late time point, the two genotypes had demonstrated clear divergence of their microbiota by principal coordinate analysis. Together, we see that there is a moderate shift in *EdnrB*-het microbiota composition over time, but no significant change in the *EdnrB*-null microbiota composition. These findings are consistent with the clustering analyses (Figure 2).

Microbiome Diversity and Richness

The alpha diversity of the microbiome was assessed using three methods. Rarefaction was used to estimate the number of operational taxonomic units (OTUs, in this case observed genera) at the genera level as an estimate of diversity (Figure 4A). The Chao1 Index was used to calculate the maximum number of OTUs as a measure of richness (Figure 4B). Finally, the Shannon-Weaver Index was used to calculate diversity and evenness (Figure 4C). As seen in the curves for Figures 4A and 4B, comparison of diversity indices is

difficult because the calculated indices are changing based on sampling effort and the curves have not reached a plateau [38]. However, the Shannon-Weaver Index plot (Figure 4C) reaches a plateau and suggests that the diversity of the microbiota decreases over time in the *EdnrB*-het animals, while the *EdnrB*-null animals display high diversity early, which persists at the later time point.

Small intestinal tissue and lumen sPLA₂ levels

To date, the majority of studies on HSCR and HAEC have focused their investigations on the colon since it is the site of aganglionosis. However, there is increasing evidence that ENS abnormalities in HSCR extend beyond the aganglionic region, and the concept is emerging that the ganglionated bowel in HSCR may in fact be abnormal [39,40]. In addition, genes that regulate ENS formation may also be involved in development of mucosal immunity [24]. Therefore, we decided to investigate whether there were changes in mucosal immunity in the ileum of *EdnrB*-null animals. Bacterial populations in the gut are regulated by antimicrobial proteins secreted by the mucosa and Paneth cells are the primary source of antimicrobial compounds for epithelial protection, releasing multiple molecules including sPLA₂, lysozyme, RegIII- γ , ang4, and cryptidins [41]. Of these, we examined expression of sPLA₂ because it is constitutively expressed along the murine GI tract [41].

Similar levels of sPLA₂ expression were detected by immunohistochemistry in both *EdnrB*-het and *EdnrB*-nulls at the early time point (P16) (Figure 5A). In contrast, at the late time point (P22), Paneth cells were still present in both groups as shown by PAS staining and *EdnrB*-hets continued to express high levels of sPLA₂ (Figure 5A) while sPLA₂ expression was no longer detected in *EdnrB*-null animals (Figure 5B). We also compared the luminal level of sPLA₂ in small intestinal wash fluid samples at P16–18 and P21–24 (Figure 5C). At P16–18, sPLA₂ activity was equivalent in *EdnrB*-het and *EdnrB*-null animals, however, by the late time point (P21–24), sPLA₂ activity was significantly decreased in the *EdnrB*-null animals compared with the *EdnrB*-het animals (2817 ± 600 versus 4887 ± 423 FL/min/ μ L, $p=0.009$). Together, these results suggest that impairment of host defense may precede or accompany the development of enterocolitis in *EdnrB*-nulls.

EVISC and Bacterial Enteroinvasion

To determine if the altered microbiome composition and expression of sPLA₂ in *EdnrB*-null animals were correlated with functional differences in susceptibility to bacterial enteroinvasion, segments of ileum from *EdnrB*-hets and *EdnrB*-nulls at the late time point (prior to enterocolitis) were challenged with enteroinvasive *E. coli*. *E. coli* was chosen because of clinical reports of its possible role in HAEC [42] as well as the finding of increased *Escherichia* in *EdnrB*-null vs. -het animals at the P21–24 time point (Table 1). Ileal segments from *EdnrB*-nulls contained more bacteria and thus were significantly more susceptible to bacterial enteroinvasion than *EdnrB*-hets ($42,400 \pm 11,582$ versus $10,133 \pm 2064$ recovered CFUs/segment, $p < 0.04$) (Figure 6A). In addition, consistent with the previous result of loss of luminal sPLA₂ activity, ileum segments from *EdnrB*-nulls released significantly less sPLA₂ in response to the presence of bacteria in culture than *EdnrB*-hets (561 ± 160 versus 1330 ± 192 FI/min/ μ L, $p < 0.01$) (Figure 6B). These findings suggest a

functional defect in antimicrobial defense associated with increased mucosal susceptibility to enteroinvasion in the ileum of *EdnrB*-null animals at the later time point.

DISCUSSION

Here we show that *EdnrB*-null animals demonstrate a shift in microbiome composition compared to *EdnrB*-hets prior to demonstrating robust colitis and peak mortality. In addition to dysbiosis, *EdnrB*-nulls exhibit altered small intestinal barrier function with the loss of expression and secretion of sPLA₂ from Paneth cells, and increased susceptibility to enteroinvasive *E. coli* compared to *EdnrB*-het animals. These data are the first demonstration of defective small intestinal mucosal antimicrobial defense associated with the development of HAEC.

In this study we utilized a clinically-relevant murine model of HSCR in which *EdnrB* is conditionally deleted from NCC [26]. These mice phenocopy the distal colonic aganglionosis typically observed in humans, display clinical manifestations of enterocolitis between the third and fourth week of life, and exhibit high mortality in the fourth week of life. We examined the microbiome composition of cecal content samples from *EdnrB*-het and *EdnrB*-null animals at an early time point (P16–18) and a later time point (P21–24), preceding the development of robust enterocolitis and mortality. Our data share some key similarities and differences with the recently-reported microbiome analysis of another model of HSCR/HAEC, the conventional *EdnrB* knockout mouse [6]. In that study, Ward, *et. al.* employed a similar approach by focusing on the development of the mucosa-associated and fecal microbiome prior to the onset of HAEC. While a direct data comparison is not possible because of differences in chosen time points for analyses, they observed similar changes at the phylum level, with decreased *Firmicutes*, and increased *Bacteroidetes* and *Proteobacteria* in the mutant animals. Unlike their findings, we observed clear differences in the composition of the microbiome of *EdnrB*-het versus *EdnrB*-null phenotype based on multiple levels of taxonomic analysis (Figure 2).

The lowest level of phylogenetic clustering from which we were able to draw meaningful conclusions was the genus. Here, we noted a decrease in *Lactobacillus* in the *EdnrB*-null animals over time while the *EdnrB*-het animals demonstrated an increase (Figure 2). This result recapitulates that seen in human HSCR patients, who also display decreased levels of *Lactobacillus* [7]. In their animal model of HSCR, Ward, *et. al.* also noted a decrease in *Lactobacillus*, although they observed this decrease in both wild type and mutant animals, concluding that weaning of the animals may contribute to the reduction of these bacteria specialized for lactose metabolism. However, their data demonstrate a marked reduction in *Lactobacillus* prior to the time of weaning, a result similar to our observation in *EdnrB*-Het animals. In an attempt to mitigate dietary effects on the microbiota in these experiments, we purposefully did not wean our animals. However, both groups of animals did have access to solid chow and, by the late time point we have observed a weight difference between the *EdnrB*-het and -null animals, suggesting that access to solid chow may contribute to the alterations of the microbiota that we observed.

We additionally observed high levels of *Bacteroides* and *Clostridium* in *EdnrB*-null animals as compared to *EdnrB*-hets at the later time point. Elevated levels of *Escherichia* were detected in *EdnrB*-null samples at the late time point that were not present in *EdnrB*-hets. While the contributions of single genera or species to the development of HAEC is doubtful, these results demonstrate that *EdnrB*-null animals exhibit dysbiosis relative to *EdnrB*-het mice, a result which may underlie the pathogenesis of HAEC.

Reductions in the levels of “protective” bacterial strains and increased levels of “pro-inflammatory” strains have been reported in patients with the inflammatory bowel disease, Crohn’s disease [43,44]. In particular, *Bacteroides* species and enteroinvasive *E. coli* have been detected at inflammatory mucosal sites and have been implicated in the pathogenesis of Crohn’s disease [44,45]. Despite these similarities, Crohn’s disease is also associated with reduced bacterial community diversity [46], while our results demonstrate a decrease in the diversity in *EdnrB*-het animals, but a high diversity in the *EdnrB*-null animals that is persistent over time. We also observed under- and over-representation of certain bacterial taxa in *EdnrB*-null mice. This difference suggests that the mechanisms by which the microbiota contributes to colitis in HSCR may be distinct from those in Crohn’s disease.

Alterations in the composition of the microbiome do not necessarily predispose humans to disease, rather dysbiosis of the microbiome together with a coexisting host susceptibility is typically required [46]. Bacterial invasion across the colonic epithelium is normally prevented by goblet cell secretion of mucus, predominantly composed of glycosylated proteins of the MUC family [47]. This intestinal barrier prevents the adherence of pathologic organisms to enterocytes and thereby reduces the susceptibility of the host to infection. Indeed, intestinal barrier function within the colon is compromised in HAEC patients by decreased secretion of mucin and increased adherence of organisms including *E. coli* and *Clostridium difficile* to enterocytes [47–52]. Interestingly, while we did not *a priori* evaluate goblet cells or mucin production, we have incidentally noted increased goblet cell size in the *EdnrB*-null animals as compared to the *EdnrB*-het animals at the later time point (Figure 5B), consistent with descriptions in HSCR patients.

Since HAEC affects both large and small bowel, and recent studies suggest that *Ret* and *EdnrB* are involved in the development of gut mucosal immunity [24,53], we investigated whether changes in the barrier function extended into the small intestine in *EdnrB*-null animals. The small intestinal surface is protected by Paneth cell secretion of antimicrobial compounds including, lysozyme, sPLA₂, RegIII γ and defensins/cryptidins [16–18,51,52]. sPLA₂ is bactericidal against gram-positive and gram-negative bacteria and functions specifically to catalyze the cleavage of fatty acids from phospholipids in cellular membranes thereby inducing bacterial membrane permeability and lysis [52]. We observed reduced Paneth cell sPLA₂ expression in ileal tissue and decreased sPLA₂ activity in the lumen of *EdnrB*-null animals compared to *EdnrB*-hets. Strikingly, tissue segments from P21–24 *EdnrB*-null mice were also four times more susceptible to bacterial enteroinvasion than *EdnrB*-hets. A similar finding of increased susceptibility to bacterial invasion is also reported in models of inflammatory bowel disease and necrotizing enterocolitis [54,55]. Additionally, small intestinal segments from *EdnrB*-nulls released significantly less sPLA₂ in response to bacterial challenge compared to *EdnrB*-het animals. These results suggest that

the gastrointestinal defects in Hirschsprung's disease are not be limited to the absence of ganglia within the colon but that mucosal immune defects extend proximally into the ganglionated bowel including the distal small intestine.

While several studies support the significance of host genotype in determining the composition of the microbiota [56,57], it is important to note that the change in microbiome composition we observed between the *EdnrB*-het and *EdnrB*-null animals at the late time point could be a secondary consequence of the colonic obstruction in the *EdnrB*-nulls rather than a direct phenotypic effect of loss of *EdnrB* signaling in the neural crest. Additionally, we cannot exclude the possibility that functional obstruction within these animals affects the quantity of food intake and contributes to the observed microbiome changes. Furthermore, while we did not see a robust inflammatory infiltrate in the P21 *EdnrB*-null bowel, there were occasional neutrophils present in some of the animals that we examined, and it is reasonable to assume that there is a gradual increase from this time point to the time of mortality. We are not able to conclude from this study if the inflammation seen is a consequence of the dysbiosis, or if the reverse is true. Measurements of temporal changes in the microbiome within individual animals, while not straightforward in our animal model due to the failure of *EdnrB*-nulls to pass stool pellets, could provide more insight into the development of HAEC within these animals. Such an approach has enabled the identification of similar patterns of microbial species during different episodes of HAEC within a single patient, thereby directly facilitating treatment regimens for HAEC [5].

Currently there are no specific therapies for HAEC [4]. Probiotics are utilized therapeutically in humans with other inflammatory diseases of the bowel, including Crohn's disease and necrotizing enterocolitis [58,59]. Theoretically, treatment of *EdnrB*-nulls with probiotics or targeted antibiotic therapy may restore the equilibrium between bacterial species, possibly preventing or delaying the development of HAEC. Supporting this, administration of a liquid diet with oral antibiotics to conventional *EdnrB* mutant mice delays, but does not prevent, HAEC onset [37]. Unfortunately, a recently completed prospective trial demonstrated no reduction in the incidence of post-operative HAEC in a group of human HSCR patients [60]. However, this study was underpowered based on the observed versus expected HAEC incidence. Furthermore, alternative probiotic formulations to the one used in the trial may yield differing results.

Together our results suggest that the ENS defects in HSCR are associated with changes in mucosal immunity and these defects are not limited to the aganglionic region of the colon but extend proximally, even into the ganglionated small intestine. Additionally, *EdnrB*-null animals display impaired mucosal barrier function and develop dysbiosis prior to the onset of HAEC, providing a reproducible model for study of this disease. These findings further our understanding of the multifactorial etiology of HAEC and may facilitate the development of strategies for both prevention and treatment.

Acknowledgments

We would like to thank Dr. Jess Reed, Department of Animal Science at the University of Wisconsin, for the generous gift of *E. coli* 5011-Lux used in the enteroinvasion studies. We would also like to thank Drew Roenneburg, Department of Surgery Histology Core, Dr. Kristina Matkowskyj and Sally Drew, Department of

Pathology and Laboratory Medicine, and Jessica Muhlenbeck for assistance with tissue processing, histology, pathological analysis and imaging.

FUNDING

This work was supported by the National Institutes of Health (NIDDK RO1DK081634) to MLE, the Veteran's Affairs Office of Research and Development (Biomedical Laboratory Research & Development Award I01BX001672) to KAK, the Central Surgical Association Foundation Turcotte Award to AG, and the American Pediatric Surgery Association Foundation Award to AG. The contents of this article do not represent the views of the Veterans Affairs or the United States Government (KAK).

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Figure 1. Survival of *EdnrB*-null animals and histologic evidence of enterocolitis

(A) *EdnrB*-null animals were followed until moribund and then sacrificed. Survival in days is plotted and the mean (28.2 days) and median (29 days) survival are indicated. (B) H&E staining of P21 and P26 *EdnrB*-het and -null colon samples (20x magnification). Black arrows indicate neutrophil infiltration in the P26 *EdnrB*-null samples. Insert shows higher magnification of neutrophils in the P26 *EdnrB*-null colon. Scale bar = 100 μ m.

Figure 2. Hierarchical clustering and ANOVA analysis of *EdnrB*-het and -null Phyla and Genera (A) Ward's minimum variance cluster analysis was employed to compare the microbiota composition of *EdnrB*-het and -null animals at the phylum level. First order branching (semi-partial R-squared=0.6119) separated a group containing 45% *EdnrB*-null Late, 30% *EdnrB*-null Early and 25% *EdnrB*-het Early (upper group), from a group containing 46% *EdnrB*-het Late, 21% *EdnrB*-null Late, 18% *EdnrB*-het Early and 14% *EdnrB*-null Early (lower group). (B) The top three phyla found at the early time point were analyzed between groups. The *EdnrB*-hets demonstrated increased *Firmicutes* and decreased *Bacteroidetes* over time (* $p < 0.05$). *EdnrB*-nulls displayed fewer *Firmicutes* than *EdnrB*-hets at both the early and late time points (* $p < 0.05$). Additionally, *EdnrB*-nulls had increased representation of *Bacteroidetes* and *Proteobacteria*, as compared to *EdnrB*-hets at the later time point (* $p < 0.05$). (C) Ward's minimum variance cluster analysis was employed to compare the microbiota composition of *EdnrB*-het and -null animals at the genus level. First order branching (semi-partial R-squared=0.4886) separated a group containing 45% *EdnrB*-null Late, 27% *EdnrB*-null Early and 27% *EdnrB*-het Early (upper group), from a group containing 79% *EdnrB*-het Late, 14% *EdnrB*-het Early and 7% *EdnrB*-null Early (lower group). (D) The top three genera found at the early time point were analyzed between groups. *EdnrB*-het animals demonstrate increased *Lactobacillus* over time, while *EdnrB*-null animals demonstrate a decrease (* $p < 0.05$). Additionally, At the late time point, *EdnrB*-null animals demonstrate decreased *Lactobacillus* and increased *Bacteroides* and *Clostridium* as compared to *EdnrB*-het animals (* $p < 0.05$).

Figure 3. Principal coordinate analysis of Unifrac distances for sequences obtained from *EdnrB*-het and *EdnrB*-null animals at early and late time points

2D representation of the first two principal coordinates. Axis percentages indicate the amount of variability explained by each coordinate. 95% confidence interval ellipses of *EdnrB*-het early (blue) and *EdnrB*-null early (yellow) animals demonstrate similarity of the flora as determined by overlapping confidence intervals. 95% confidence interval ellipses of *EdnrB*-het late (green) and *EdnrB*-null late (red) animals demonstrates distinct flora as determined by non-overlapping confidence intervals. This separation appears to be based primarily on the first principal coordinate (x-axis).

Figure 4. Microbial sequence diversity

Three methods were used to assess alpha diversity of the microbiome: (A) Rarefaction (Operational Taxonomic Unit, OTUs, at a level of sequence similarity >97%). (B) Chao1 Index. (C) Shannon-Weaver Index. By two-way ANOVA with Bonferonni post-hoc analysis, *EdnrB*-het late animals differ from the other groups (* $p=0.004$).

Figure 5. Small intestinal tissue sPLA₂ expression and wash fluid sPLA₂ activity

(A) Representative immunohistochemical images of Paneth cell sPLA₂ (red) with DAPI cell nucleus stain (blue) in ileal segments. sPLA₂ was expressed at the early time point (P16) in both animal groups, but was not visible in *EdnrB*-nulls at the late time point (P22) (B) PAS staining demonstrating that Paneth cells (black arrows) appear to be present in normal numbers in *EdnrB*-null animals at the later time point. (C) At P16–P18, *EdnrB*-het and *EdnrB*-null animals showed similar luminal levels of sPLA₂ activity. However, by P21–24, *EdnrB*-null mice had significantly decreased luminal levels of sPLA₂ activity compared to *EdnrB*-hets (* $p < 0.01$). Scale bars = 100 μ m.

Figure 6. Recovered bacteria from tissue segments and sPLA₂ activity in secretions after *ex vivo* intestinal segment culture (EVISC)

(A) *EdnrB*-null ileal segments had significantly more enteroinvasive *E. coli* recovered compared to *EdnrB*-het, demonstrating increased enteroinvasive susceptibility (* $p < 0.05$). (B). sPLA₂ activity from secretions of ileal segments during enteroinvasion challenge was significantly decreased in *EdnrB*-null animals compared to *EdnrB*-hets (* $p < 0.05$).

Table 1

Relative percentages of top ten genera in cecal fecal samples from *EdnrB*-het and *EdnrB*-null at the Early (P16–18) and Late (P21–24) time points. Values represent the mean $\pm$ SEM. Means without a common superscript letter are significantly different ($p < 0.05$). NS denotes no significant differences between groups. Differences were assessed using ANOVA with Tukey HSD post-hoc analysis.

Genus	EdnrB-Het Early	EdnrB-Null Early	EdnrB-Het Late	EdnrB-Null Late
<i>Lactobacillus</i>	52.41 $\pm$ 4.35 ^A	39.11 $\pm$ 5.69 ^A	79.19 $\pm$ 5.36 ^B	14.08 $\pm$ 3.59 ^C
<i>Bacteroides</i>	9.71 $\pm$ 2.95 ^{AB}	16.89 $\pm$ 5.14 ^A	0.20 $\pm$ 0.06 ^B	15.91 $\pm$ 3.77 ^A
<i>Blautia</i>	7.43 $\pm$ 1.80 ^{AB}	5.33 $\pm$ 1.63 ^{AB}	2.45 $\pm$ 0.80 ^B	11.82 $\pm$ 2.75 ^A
<i>Clostridium</i>	7.27 $\pm$ 1.86 ^{AB}	7.49 $\pm$ 2.10 ^{AB}	2.76 $\pm$ 0.73 ^B	12.68 $\pm$ 3.54 ^A
<i>Streptococcus</i>	4.92 $\pm$ 1.45 ^{AB}	7.70 $\pm$ 1.43 ^A	0.60 $\pm$ 0.19 ^B	3.02 $\pm$ 1.15 ^B
<i>Ruminococcus</i>	2.60 $\pm$ 1.17 ^{NS}	2.09 $\pm$ 0.88	1.04 $\pm$ 0.27	2.76 $\pm$ 0.81
<i>Staphylococcus</i>	1.87 $\pm$ 0.94 ^{NS}	0.18 $\pm$ 0.84	0.36 $\pm$ 0.14	0.76 $\pm$ 0.40
<i>Roseburia</i>	1.67 $\pm$ 0.40 ^{NS}	2.06 $\pm$ 0.84	3.03 $\pm$ 1.82	2.09 $\pm$ 0.44
<i>Allobaculum</i>	1.55 $\pm$ 1.43 ^{NS}	1.81 $\pm$ 0.84	0.70 $\pm$ 0.28	4.77 $\pm$ 3.39
<i>Eubacterium</i>	1.14 $\pm$ 0.46 ^A	1.56 $\pm$ 0.51 ^A	2.33 $\pm$ 0.75 ^A	10.67 $\pm$ 2.92 ^B