Enteric Glia Regulate Gastrointestinal Motility but are not Required for Maintenance of the Epithelium in Mice

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Abstract

Background & Aims—When the glial fibrillary acidic protein (GFAP) promoter is used to express cellular toxins that eliminate glia in mice, intestinal epithelial permeability and proliferation increase; this led to the concept that glia are required for maintenance of the gastrointestinal epithelium. Many enteric glia, however, particularly in the mucosa, do not express GFAP. In contrast, virtually all enteric glia express proteolipid protein 1 (PLP1). We investigated whether elimination of PLP1-expressing cells compromises epithelial maintenance or gastrointestinal motility.

Methods—We generated mice that express tamoxifen-inducible Cre recombinase under control of the *Plp1* promoter and carry the diptheria toxin subunit A (DTA) transgene in the Rosa26 locus (Plp1^CreER;Rosa26^DTA mice). In these mice, PLP1-expressing glia are selectively eliminated without affecting neighboring cells. We measured epithelial barrier function and gastrointestinal motility in these mice and littermate controls, and analyzed epithelial cell proliferation and ultrastructure from their intestinal tissues. To compare our findings with those from previous studies, we also eliminated glia with ganciclovir in Gfap^HSV-TK mice.

Results—Expression of DTA in PLP1-expressing cells selectively eliminated enteric glia from the small and large intestines, but caused no defects in epithelial proliferation, barrier integrity, or

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ultrastructure. In contrast, administration of ganciclovir to Gfap[HSV-TK] mice eliminated fewer glia but caused considerable non-glial toxicity and epithelial cell death. Elimination of PLP1-expressing cells did not reduce survival of neurons in the intestine, but altered gastrointestinal motility in female, but not male, mice.

Conclusions—Using the *Plp1* promoter to selectively eliminate glia in mice, we found that enteric glia are not required for maintenance of the intestinal epithelium but are required for regulation of intestinal motility in females. Previous observations supporting the concept that maintenance of the intestinal epithelium requires enteric glia can be attributed to non-glial toxicity in Gfap[HSV-TK] mice and epithelial-cell expression of GFAP. Contrary to widespread notions, enteric glia are therefore not required for epithelial homeostasis. However, they regulate intestinal motility in a sex-dependent manner.

Keywords

enteric nervous system; epithelial barrier; sex differences

Introduction

The enteric nervous system (ENS) is a large network of neurons and glia intrinsic to the alimentary tract that is essential for gastrointestinal homeostasis[1]. While it is now well recognized that glial dysfunction contributes to neurological disorders[2], the role of glia in digestive disorders remains unclear. Understanding normal enteric glial function is essential for identifying glial contributions to digestive disease. Enteric glia are distributed throughout the laminar structure of the gastrointestinal tract and closely appose neurons, immune cells, blood vessels and the intestinal epithelium[3–5]. Mucosal glia in the lamina propria directly underlie the epithelium and encircle crypts of Lieberkühn, which contain the epithelial stem cell niche[6]. This close proximity raised the possibility that enteric glia play a role in regulating epithelial functions such as cellular proliferation and barrier maintenance.

A series of studies that investigated the consequences of eliminating GFAP-expressing glia in mice suggested that enteric glia may be essential for epithelial maintenance and offered clues to glial functions in the bowel. Initial studies used mice that express herpes simplex virus thymidine kinase (HSV-TK) under the control of the *Gfap* promoter (Gfap[HSV-TK] mice). Upon systemic treatment of Gfap[HSV-TK] mice with ganciclovir, GFAP-expressing enteric glia were eliminated and the animals developed fulminant inflammation selectively in the mid-to-distal small intestine[7]. Intestinal epithelial permeability and crypt cell proliferation were increased even before histological evidence of inflammation was detected[8]. These observations were interpreted to mean that enteric glia are required for normal regulation of barrier function and epithelial turnover, and that elimination of enteric glia causes epithelial dysregulation, leading to translocation of luminal bacteria and intestinal inflammation[7]. Subsequent studies using mice engineered to manifest autoimmune reactions against GFAP-expressing cells also reported intestinal inflammation and premature death[9]. *In vitro*, permeability of Caco-2 cell monolayers and their incorporation of [3H]-thymidine both decrease when these cells are co-cultured with enteric glia[8, 10], further suggesting that enteric glia can regulate epithelial permeability and proliferation. These experiments have led to the concept, now widely accepted, that enteric glia directly regulate

important bowel functions independently of their interactions with neurons and to speculation that enteric glial dysfunction contributes to the pathogenesis of inflammatory bowel disease[11].

More recent studies have shown that enteric glia are heterogeneous and that GFAP expression is limited to a subset[12, 13]. More than half of mucosal and submucosal glia in the ileum and colon do not express GFAP[13]. In contrast, S100β, SOX10 and PLP1 are expressed by virtually all enteric glia and the *Plp1* promoter can be used to manipulate enteric glia *in vivo*[13]. To explore enteric glial functions, we targeted PLP1-expressing cells for elimination in mice using inducible expression of diphtheria toxin subunit A (DTA). We generated Plp1^{CreER};Rosa26^{DTA} mice that express tamoxifen-inducible Cre recombinase (Cre) under the control of the *Plp1* promoter and carry the lox-stop-lox-DTA transgene in the Rosa26 locus. Administration of tamoxifen induces DTA expression, selectively eliminating PLP1-expressing glia without affecting neighboring cells. The robust elimination of enteric glia induced in tamoxifen-treated Plp1^{CreER};Rosa26^{DTA} mice revealed that enteric glia are dispensable for all epithelial functions examined but are required for normal intestinal motility.

Methods

Mouse lines

Plp1^{CreER}, Rosa26^{DTA}, Gfap^{CreER}, Rosa26^{TdTomato}, Rosa26^{LacZ}, Gfap^{Cre} and Gfap^{HSV-TK} mice are detailed in Supplemental Table 1. Plp1^{CreER} mice were on a FVB background and others were on a C57BL6 background. Plp1^{CreER};Rosa26^{DTA} (Cre$^+$, experimental) and Rosa26^{DTA} (Cre$^−$, control) mice used for all experiments were littermates on a mixed FVBN:C57BL6 background. Mice were housed in a facility with a 12h dark cycle and handled in accordance with Columbia University IACUC guidelines.

Induction of glial loss

Plp1^{CreERT};Rosa26^{DTA} (Cre$^+$) and Rosa26^{DTA} mice (Cre$^−$) were gavaged once at 5–6 weeks of age with 8mg of tamoxifen (Toronto Research Chemicals T006000) solubilized in corn oil. All measurements were obtained at 7–9dpt unless noted. GFAP^{HSV-TK} mice were treated with ganciclovir via SQ pump or gavaged with 50mg/kg of valganciclovir (Cayman Chemical 13875) twice daily for up to 15 days starting at 5–6 weeks of age. We observed no difference between these two induction methods and the data presented are from mice treated with valganciclovir.

Epithelial permeability and proliferation

For permeability measurements, mice were fasted overnight and gavaged with 600mg/kg FITC-dextran (4 kDa, Sigma FD40S) or 200mg/kg fluorescein-5-(and-6)-sulfonic acid (478 Da, Invitrogen F-1130). Four hours later, serum was collected and fluorescence intensity was measured as previously described[8]. For dextran sodium sulfate (DSS) colitis, mice were treated with 2.5% DSS (Affymetrix 14489) in drinking water for 7 days. Body weight and disease activity index (composite of stool consistency and hematochezia) were assessed daily. For measurement of bacterial penetration into mesenteric lymph nodes (MLNs), RNA

was extracted from MLNs and reverse-transcribed. Quantitative PCR (SYBR green) was performed for mouse *Gapdh* and universal primers for bacterial 16S rRNA[14]. $C_T=C_T(16S)-C_T(Gapdh)$. For analysis of epithelial proliferation at 7dpt, villus-to-crypt ratios were measured in hematoxylin and eosin-stained paraffin sections (5μm), and represent maximum villous length divided by maximum crypt length. Thirty villus-crypt units were measured from each animal per intestinal segment. Ki67 immunoreactivity was quantified in 5μm paraffin sections. At least 50 crypts/intestinal segment were measured per animal.

Immunocytochemistry, microscopy and quantification

Immunostaining was performed as previously described[13]. Primary antibodies are detailed in Supplemental Table 2. Anti-goat and anti-donkey secondary antibodies used were conjugates of AlexaFluor 594, 568 or 488 (Invitrogen). Nuclei were counterstained with DAPI (Vector Labs H-1200) or TO-PRO3 (Molecular Probes T3605). For glial density measurement, segments of ileum and colon were immunostained for S100β and NF-H as previously described[13], and single planar images were captured at the level of the myenteric plexus or mucosa (5–6 fields per segment identified based on NF-H staining alone). Mucosal glia were quantified relative to number of glands. Images from all animals were coded, randomized and quantified using ImageJ or Volocity 6.3 (PerkinElmer) by a blinded investigator. To quantify neuronal density, segments of ileum and colon were immunostained for ANNA-1 or nNOS and quantified as above. For transmission electron microscopy (TEM), tissues were fixed in a mixture of 4% formaldehyde and 2.5% glutaraldehyde, phosphate-buffered to pH7.4 and post-fixed with 1% OsO_4. Fixed tissue was stained with 1% uranyl acetate in maleate buffer (0.5%; pH 6.2), dehydrated with ethanol, cleared with propylene oxide, and embedded in an epoxy resin (Epon812). Thin sections were cut with an ultramicrotome, placed on formvar-coated nickel slot grids, stained with uranyl acetate (1%) and Reynolds lead citrate, and viewed with a JEOLCO TEM (JEM 1200).

Gastrointestinal motility

Total gastrointestinal transit time, gastric emptying, colonic motility, and small intestinal transit were measured as previously described[15]. Small intestinal transit was estimated by the position of the geometric center of rhodamine-dextran 15 minutes after gavage[16]; values are distributed between 1 (minimal motility) and 10 (maximal motility). For fecal composition measurements, mice were transferred to empty cages at 9AM and stool was collected for 1 hour to measure "wet" stool weight per mouse. Stool was dried at 45°C for 48 hours and then "dry" weight was recorded. % water content=[("wet"-"dry")/# pellets produced in 1 hour]*100.

Statistics

Student's unpaired *t* tests were used to compare two means. Two-way ANOVA with Sidak's multiple comparisons testing was used to compare multiple means in DSS experiment. Graphs display mean ± standard error of the mean (SEM) with all individual data points shown. Each data point represents an individual mouse. A *p* value of less than 0.05 was considered significant and denoted with *. *P* values less than 0.005 are noted with ** and less than 0.0005 with ***.

Results

DTA expression in PLP1⁺ cells provides an inducible, non-inflammatory method to eliminate enteric glia

Administration of a single dose of tamoxifen by oral gavage to Plp1^{CreER};Rosa26^{DTA} mice (annotated as Cre⁺) led to a 66% loss of S100β⁺ myenteric and intramuscular glia throughout the small intestine and a 74% loss in the large intestine, relative to Rosa26^{DTA} littermates (annotated as Cre⁻) within 7 days (Figure 1). All known types of enteric glia were depleted in Cre⁺ mice, including myenteric, intramuscular, submucosal and mucosal (Figure 1E–G). Mucosal glia were most severely affected with Cre⁺ mice showing 88% fewer mucosal glia in the small intestine than Cre⁻ controls (p<0.0001, n=6–7mice/group). DTA poisons cells by interfering with protein synthesis. To ensure that DTA induction caused loss of glia and not just loss of glial marker expression, ENS ultrastructure was examined. In Cre⁺ mice, the glial sheath that normally separates axons within nerve fiber bundles from each other and from surrounding connective tissue was lost, enabling collagen fibers to penetrate the enteric plexuses and directly contact axons (Figure 1J–K). Enteric glial elimination was tamoxifen-dependent; Cre⁺ mice treated with vehicle exhibited no glial loss and were indistinguishable from Cre⁻ littermates (Figure S1). These data suggest that Plp1^{CreER};Rosa26^{DTA} mice provide a robust, inducible, cell-specific mouse model for studying enteric glial function *in vivo*. Schwann cells in the peripheral nervous system (PNS) and oligodendrocytes in the central nervous system (CNS) also express PLP1[17, 18] and mice with inducible expression of DTA within PLP1-expressing cells have been reported to model CNS demyelination[19]. Consistent with that report, the Plp1^{CreER};Rosa26^{DTA} mice utilized in our study also developed hindlimb weakness and coordination deficits starting at 14 days post-tamoxifen induction (dpt); therefore, we limited all analysis of gastrointestinal function to the first 10dpt. Glial loss was similar at 5, 7, 9 and 14dpt in Plp1^{CreER};Rosa26^{DTA} mice, suggesting that the enteric glial deficit was stable during the period of analysis.

Remarkably, the fulminant inflammation in the mid-to-distal small intestine that occurs when GFAP-expressing cells are eliminated[7] did not occur when PLP1-expressing cells were eliminated. Although PLP1 is expressed by far more enteric glia than GFAP and GFAP-expressing glia were clearly eliminated in Plp1^{CreER};Rosa26^{DTA} mice (Figure 1H), no gross or histological evidence of inflammation in the small or large intestine was observed at any time point examined from 48h to 14dpt (Figures 1M–N, S2). These data suggest that inflammation is not an inevitable consequence of enteric glial elimination.

Enteric glia do not regulate epithelial permeability or homeostasis

Studies based on elimination of GFAP-expressing cells led to the idea that enteric glia regulate intestinal permeability and epithelial turnover[8, 20]. Because mice in these studies developed intestinal inflammation, which can independently compromise the epithelial barrier and alter epithelial cell proliferation, a direct link between glial elimination and epithelial dysfunction was not tested. Plp1^{CreER};Rosa26^{DTA} mice exhibit robust glial loss in the absence of inflammation, providing a means to investigate the role of enteric glia in epithelial homeostasis without confounding effects. To assess whether glial elimination

alters macromolecular intestinal permeability, 4.4kDa FITC-dextran or a 478Da fluorescein conjugate of 5, 6-sulfonic acid was administered to Cre$^+$ and Cre$^-$ mice by oral gavage; absorption of the tracers was then measured 4 hours later. Serum levels of both tracers did not differ between control and glial-depleted mice (Figure 2A–B). In contrast, when measured in wildtype mice with acute chemical colitis induced with dextran sodium sulfate (DSS) as a positive control, serum FITC-dextran was markedly increased (Figure 2A). Enteric glial elimination in Plp1^{CreER};Rosa26^{DTA} mice, moreover, did not change 16S rRNA transcript levels in mesenteric lymph nodes (Figure 2C) indicating that microbial translocation through the intestinal epithelial barrier did not increase. Localization of the proteins zonulin-1 and E-cadherin, which are essential for normal intercellular junctions between enterocytes, was also unchanged (Figure 2D–E). Ultrastructural analysis revealed no differences between Cre$^+$ and Cre$^-$ mice in enterocyte appearance. Apical regions, brush borders and intercellular junctions of enterocytes remained intact (Figure 2F–G). To determine whether glial-depleted mice might have subtle barrier defects that sensitize them to inflammatory stress, acute DSS colitis was induced in Cre$^+$ and Cre$^-$ mice starting at 4dpt. All mice developed colitis over the course of 7 days, and there was no difference in weight loss or disease activity between Cre$^+$ and Cre$^-$ mice (Figure 2H–I), implying that glial loss does not sensitize mice to intestinal inflammation. In sum, these data suggest that enteric glia are not required for maintaining the integrity of the intestinal epithelial barrier or inhibiting inflammation.

To test the role of enteric glia in epithelial proliferation, we measured the ratio of villus height to crypt depth (V/C ratio) and Ki67-immunoreactive cells/crypt in Cre$^+$ and Cre$^-$ mice. Distinct V/C ratios are characteristic of different parts of the small intestine, and V/C ratios are altered by conditions that affect epithelial stem cell function, such as radiation injury, starvation, and inflammatory insults[21]. There was no change in V/C ratio upon induction of glial elimination, and regional distinctions in V/C ratio were maintained (Fig. 3A). Quantitation of Ki67$^+$ cells revealed that crypt cell mitosis was also not altered (Fig. 2B–D). Elimination of the majority of enteric glia throughout the intestine thus triggers no change in epithelial barrier integrity or proliferation.

GFAP^{HSV-TK} mice exhibit off-target effects leading to epithelial damage

To reconcile our observations with those previously reported in mice in which GFAP expression was used to target enteric glia, we attempted to generate Gfap^{CreER};Rosa26^{DTA} mice for direct comparison. We first characterized *Gfap* promoter-driven Cre expression by breeding Gfap^{CreER} mice with a Rosa26^{LacZ} reporter strain[22]. Minimal reporter expression was detected in the ENS upon tamoxifen treatment despite robust CNS reporter expression, consistent with previous reports of low enteric recombination efficiency in other Gfap^{CreER} mice[23]. LacZ enzymatic activity was, however, detected within occasional stripes of epithelial cells (Figure 4A), suggesting that the *Gfap* promoter is transiently active in some intestinal epithelial stem cells. Isolated TdTomato-expressing cells were also observed in the small intestinal epithelium of Gfap^{Cre};Rosa26^{TdTomato} reporter mice (Figure 4B)[24]. Consistent with these findings, rare epithelial cells in the distal small intestine were GFAP-immunoreactive (Figure 4C).

Because we were unable to identify a Gfap^{CreER} line that selectively expressed Cre in enteric glia, we characterized the original Gfap^{HSV-TK} mouse line used in prior studies[7]. A subset of enteric glia, mainly within myenteric ganglia, exhibited TK immunoreactivity in Gfap^{HSV-TK} mice (Figure 5A, D). Upon systemic treatment with subcutaneous ganciclovir or the oral pro-drug valganciclovir, GFAP-expressing enteric glia were depleted throughout the gastrointestinal tract (Figure 5B–C). Following treatment of TK$^+$ mice with valganciclovir, the ilea and colons contained respectively, 53% and 65% fewer S100β$^+$ myenteric and intramuscular glia than their TK$^-$ littermates (Figure 5E–F; n=5 mice/group). Consistent with prior reports, valganciclovir treatment led to hemorrhagic inflammation in the mid-to-distal small intestine of TK$^+$ mice evident between 6–15 days of treatment, as well as altered macromolecular epithelial permeability (Figure 5G). Prior reports attributed the restricted nature of inflammation in these mice to a presumed lack of transgene expression in the large intestine and regional differences in rate of glial turnover, but these presumptions were never verified. We detected both TK transgene expression and valganciclovir -dependent glial loss in the large intestines of Gfap^{HSV-TK} mice (Figure 5D–F), suggesting that the transgene expression pattern does not explain the regional specificity of intestinal inflammation in these mice.

Unlike DTA, the cellular toxin generated by HSV-TK from ganciclovir can diffuse into and poison neighboring cells. To determine whether non-glial cells were affected in Gfap^{HSV-TK} mice, we analyzed expression of phosphorylated-histone 2AX (pH2AX), a marker of double-stranded DNA breaks that are an early step in the apoptotic cascade[25]. Phosphorylated-H2AX immunoreactivity was widespread in small intestinal crypts in TK$^+$ mice treated with valganciclovir (Figure 5H–I). Extensive pH2AX immunoreactivity was also detected in non-TK expressing cells in the muscularis externa and myenteric plexus, suggesting that the toxicity of metabolites generated by HSV-TK is not limited to enteric glia in Gfap^{HSV-TK} mice (Figure S3A–D). In contrast, in Plp1^{CreER};Rosa26^{DTA} mice treated with tamoxifen, there was no difference in epithelial pH2AX immunoreactivity between Cre$^-$ and Cre$^+$ animals despite more extensive glial loss in Cre$^+$ than in TK$^+$ mice (Figure S3E–F). Ultrastructural analysis of small intestines from Gfap^{HSV-TK} mice treated with valganciclovir revealed abnormal appearing, vacuolated epithelial cells with disorganized microvilli and intercellular junctions, even in regions with intact mucosal glia (Figures 5J–L). These data suggest that the Gfap^{HSV-TK} model of glial elimination causes toxicity to non-glial cells. GFAP expression in rare ileal epithelial cells likely contributes to these off-target effects. This inadvertent toxicity might explain why inflammation occurs in Gfap^{HSV-TK} mice but not in Plp1^{CreER};Rosa26^{DTA} mice, in which glial elimination is cell-autonomous.

Enteric glia regulate colonic motility in a sex-dependent manner

Although elimination of PLP1-expressing cells did not affect epithelial homeostasis, it altered gastrointestinal motility selectively in females. Total gastrointestinal transit time (GITT) was shorter in female Plp1^{CreER};Rosa26^{DTA} mice than in Cre$^-$ littermates after treatment with tamoxifen; but no different in males (Figure 6A). Fecal pellet size and water content were unchanged by glial loss in both sexes (Figure 6B–C), suggesting that fecal composition was unlikely to explain the difference in transit. GITT is a composite of the

time required for gastric emptying, small intestinal and colonic transit, each of which is dependent on intrinsic enteric neuronal activity and modified by CNS input. When measured separately, enteric glial elimination did not alter the rates of gastric emptying or small intestinal transit (Figure 6D–E), suggesting that the effects of enteric glial elimination are most prominent in the colon. We therefore examined colonic motility in more detail and excluded the effects of extrinsic nerves by studying the propagation of colonic migrating motor complexes (CMMCs; an ENS-dependent manifestation of the peristaltic reflex) in an *ex vivo* preparation[15, 26]. Colons were isolated from Cre⁻ and Cre⁺ mice, suspended in oxygenated Krebs solution and perfused through the lumen at an intraluminal pressure sufficient to induce CMMCs. Intrinsic microcircuits of the ENS drive CMMCs under these conditions, which can be recorded and analyzed. All CMMCs propagated in an oral to anal direction; no retrograde propagation was observed. In males, there was no difference between Cre⁻ and Cre⁺ mice in CMMC frequency, velocity or length of propagation (Figure 6H–J). In contrast, CMMC frequency in female Cre⁺ mice was twice that of their Cre⁻ littermates (Figure 6F–H). Notably, CMMC frequency in Cre⁻ controls was higher in males than females (Figure 6H). Glial disruption selectively altered the peristaltic reflex in female mice, increasing CMMC frequency to male levels (Figure 6H). Velocity and length of CMMC propagation were both unchanged in female glial-depleted mice (Figure 6I–J), suggesting that enteric glia are specifically important for regulating CMMC frequency.

The integrity of cell types important for peristaltic reflexes was examined next. Enterochromaffin cells trigger peristaltic reflexes through the release of serotonin (5-HT)[27]. There was no difference in colonic enterochromaffin cell number between Cre⁻ and Cre⁺ mice, in males (p=0.62, n=4/group) or females (p=0.5, n=4/group). Total neuronal density and the density of nitrergic inhibitory motor neurons were also not different in Cre⁻ and Cre⁺ mice (Figure 7A–F), suggesting that altered motility was not due to an effect on neuronal survival. The nerve fiber network, furthermore, appeared grossly normal (Figure 4A–B). The network of interstitial cells of Cajal (ICCs), the pacemaker cells of the gut resident in the muscularis externa[28], also appeared intact (Figure 7G–H). Enteric glia are thus not necessary for the maintenance of enteric neurons, enterochromaffin cells or ICCs in the myenteric plexus, and the role of glia in gastrointestinal motility is probably more complex. In summary, our findings suggest that glia play an essential and sex-dependent role in the ENS regulation of gastrointestinal motility but are not required for epithelial maintenance or neuronal survival.

Discussion

Enteric glia and epithelial homeostasis

The current understanding of enteric glia is heavily influenced by studies that used the *Gfap* promoter to target cells for elimination in mice and reported intestinal inflammation. We show that using a cell-autonomous toxin to target PLP1-expressing cells eliminates an even wider population of glia, including those that express GFAP, without causing intestinal inflammation. These findings suggest that the method of disrupting enteric glia, rather than glial disruption itself, explains the previous observations of inflammation. We find that GFAP^HSV-TK mice exhibit extensive signs of non-glial toxicity, especially in the intestinal

epithelium. This is probably due to GFAP expression in rare epithelial cells of the distal small intestine and non-cell-autonomous effects of the toxin generated by HSV-TK. Findings from transgenic mouse models that utilize the *Gfap* promoter to study gastrointestinal function must be interpreted with the possibility of epithelial promoter activity in mind.

Elimination of PLP1-expressing cells using the cell-autonomous DTA approach triggered widespread, selective loss of enteric glia, yet did not induce changes in intestinal permeability, epithelial intercellular junctions, microbial translocation, enterocyte ultrastructure, or crypt cell proliferation. These observations led us to conclude that enteric glia are not required for epithelial maintenance *in vivo*. In the only previous study in which enteric glia were eliminated without selectively targeting GFAP-expressing cells (using the gliotoxin 6-aminonicotinamide), the ultrastructure of myenteric neurons and the colonic epithelium appeared normal despite extensive glial loss[29]. Similarly, studies in which investigators disrupted glial function without cell elimination also support the idea that glial dysfunction does not directly cause epithelial dysfunction (Supplemental Table 3)[30, 31]. The chemical and genetic lesions in prior studies probably disrupted some subset(s) of enteric glia more than others; moreover, none of the studies examined epithelial proliferation or *in vivo* barrier permeability. Plp1^CreER;Rosa26^DTA mice exhibit robust, inducible elimination of all types of enteric glia throughout the intestine without any evidence of epithelial defects. These data show that intestinal epithelial integrity does not depend on the presence or normal function of enteric glia in healthy mice.

Enteric glia can secrete a number of signaling molecules that modulate epithelial function *in vitro*[8, 10, 32–36] yet glia seem unnecessary for epithelial integrity *in vivo*. This difference could occur because other cell types secrete these signaling molecules in the absence of glia, or because enteric glia have different capabilities *in vitro* and *in vivo*. Such differential capabilities have been reported with regard to their neurogenic potential[23, 37]. Enteric glia, like other glia, are probably dynamic and highly sensitive to their microenvironment. Removing them from the intestinal milieu might enable them to secrete signals that they do not normally secrete *in vivo;* moreover, cultured glia might approximate some subset(s) of enteric glia better than others. Enteric glia express toll-like receptors[38], require the microbiome for migration[39], and communicate with immune cells[5]. It is thus conceivable that although enteric glia are not required for epithelial homeostasis in healthy mice, they might influence the epithelium when "activated" by certain infectious or immunological conditions. Plp1^CreER;Rosa26^DTA mice represent a new tool to test this possibility *in vivo*. Glia can adopt pro- or anti-inflammatory phenotypes depending on the context[40], and enteric glia play a role in the neurotoxic effects of intestinal inflammation[41]. Further investigations of how infectious, dietary, or genetic insults modulate glial phenotype are necessary to better understand the role of glia in epithelial physiology and pathophysiology.

Glial regulation of gastrointestinal motility

Although glial elimination in Plp1^CreER;Rosa26^DTA mice does not alter the intestinal epithelium, it does cause specific defects in gastrointestinal motility. Glial-depleted female mice, unexpectedly, had accelerated total gastrointestinal transit time and increased CMMC

frequency; similarly, mice treated with 6-aminonicotinamide develop diarrhea[29]. Studies of disrupted enteric glial function also support a role for glia in the regulation of intestinal motility, although the region of intestine affected and the direction of change vary across studies (Supplemental Table 3)[30, 42, 43] Together with our findings, these observations show that altering glial function affects gastrointestinal motility; however, the magnitude and direction of the effects are dependent on the nature of the lesion. This variability might reflect the differential effects of each of these lesions on enteric glial subtypes and on glia outside the ENS. Identifying molecular markers for enteric glial subtypes and developing approaches to disrupt enteric glia without altering extra-enteric glia will be essential to better define the role of glia in the regulation of gastrointestinal motility.

Remarkably, we found that the effects of glial elimination on intestinal motility in Plp1^{CreER};Rosa26^{DTA} mice were sex-dependent. Sex-dependent phenotypes were observed in both *in vivo* and *ex vivo* measurements of motility, suggesting that these differences cannot simply be attributed to extrinsic inputs. There were no sex differences in extent of glial elimination or in any other phenotype examined, suggesting that this finding is highly specific to glial regulation of motility. Sex differences were not examined in previous studies of enteric glial disruption; however, functional gastrointestinal disorders exhibit prominent sex differences in both prevalence and phenotype that remain largely unexplained[44]. Differences in glial regulation of motility could explain some of these observations.

Many cell types in the gut play crucial roles in motility, including enterochromaffin cells, enteric neurons, muscularis macrophages, sympathetic inputs, and the 3 cell types that comprise the SIP syncytium (smooth muscle cells, ICCs, and platelet-derived growth factor receptor α^+ cells)[45]. The sex-dependent defect in gastrointestinal motility observed upon glial elimination could be a direct effect resulting from sex differences in enteric glia themselves, or an indirect effect arising from sexual dimorphism in any of the other cell types that interact with glia. Enterochromaffin cells vary in number over the course of the estrus cycle[26] and enteric glia closely appose enteroendocrine cells[3]. Although glial elimination in Plp1^{CreER};Rosa26^{DTA} mice did not affect enterochromaffin cell density in either sex, it remains possible that glia modulate 5-HT release or other aspects of enterochromaffin cell function that are sex-dependent. Some enteric neurons express estrogen receptors[46] and intestinal smooth muscle cells express androgen receptors[47], suggesting that sex hormones signal to cell types important for motility. Plp1^{CreER};Rosa26^{DTA} mice offer a robust tool to interrogate these intercellular interactions. Glia are considered essential for neuronal health and yet enteric neurons in Plp1^{CreER};Rosa26^{DTA} mice appeared remarkably intact and gastrointestinal motility in male mice was unchanged. While glia may not be essential for enteric neuronal survival in healthy mice, their absence may have other consequences for neurons. Electrophysiological studies of Plp1^{CreER};Rosa26^{DTA} mice as well as development of longer-term models of glial loss will be helpful to analyze these consequences.

In summary, genetic ablation of the majority of enteric glia does not alter intestinal epithelial proliferation, barrier function or ultrastructure but does lead to a sex-dependent defect in colonic motility. Previous findings using GFAP^{HSV-TK} mice to support a role for glial regulation of the epithelium were probably confounded by toxicity to neighboring non-glial

cells. These findings change the current paradigm for understanding the role of enteric glia in digestive physiology and pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Author names in bold designate shared co-first authorship.

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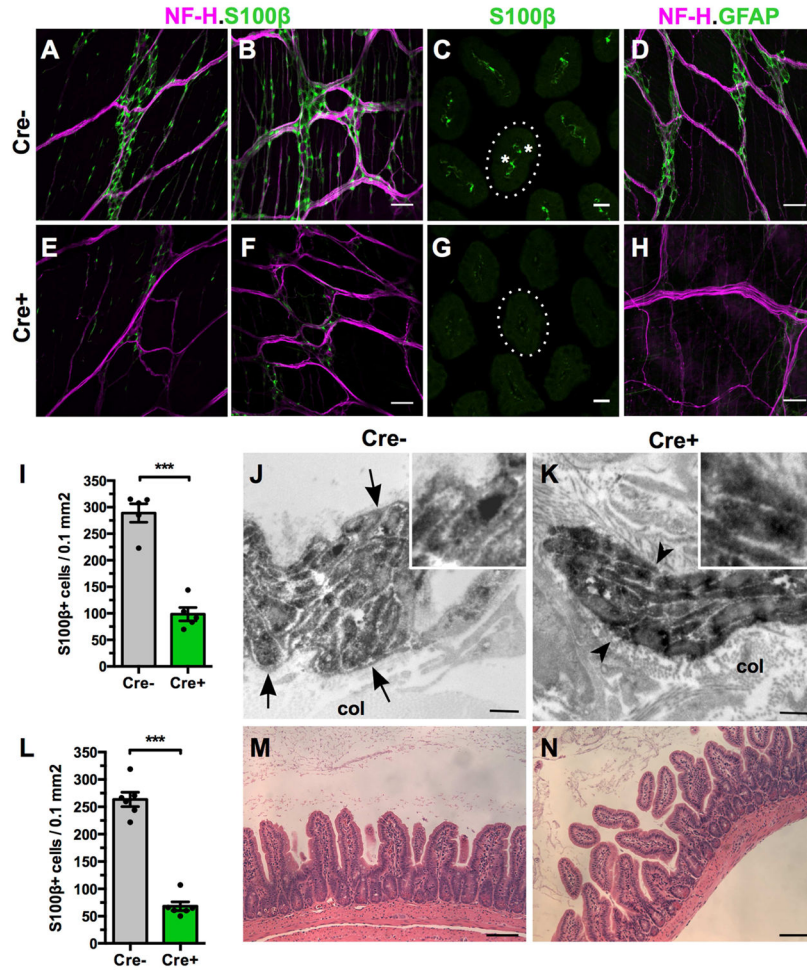
Figure 1. DTA expression in PLP1-expressing cells leads to enteric glial loss without associated inflammation

Neurofilament-H (NF-H) and S100β immunoreactivities in the ilea (A, E) and colons (B, F) of Rosa26^{DTA} (Cre$^-$, control) and Plp1^{CreER};Rosa26^{DTA} mice (Cre$^+$, experimental) reveal Cre-dependent loss of myenteric and intramuscular glia.

(C, G) Ileal S100β immunoreactivity, imaged at the level of the villous mucosa, highlights the loss of mucosal glia (*) in the lamina propria of Cre$^+$ mice. Cross-sections of single villi are outlined.

(D, H) NF-H and GFAP immunoreactivities show the Cre-dependent loss of ileal GFAP$^+$ myenteric glia.

(I, L) Quantitation of S100β-expressing cells in ileum (I) and colon (L).

(J–K) Electron microscopic visualization of ileal PGP9.5 immunoreactivity (neuronal marker). In the Cre$^-$ control, a glial sheath (arrows) separates neuronal processes from the surrounding collagen (col). Thin glial partitions are seen between bundles of immunoreactive axons (see inset). In contrast, in Cre$^+$ tissue there is no glial sheath around axon bundles or glial partitions separating axons. Collagen fibers thus come into direct contact with axons (arrowheads).

(M–N) Hematoxylin and eosin stained cross-sections of ilea from Cre⁻ (M) and Cre⁺ mice (N) reveal no evidence of inflammation.

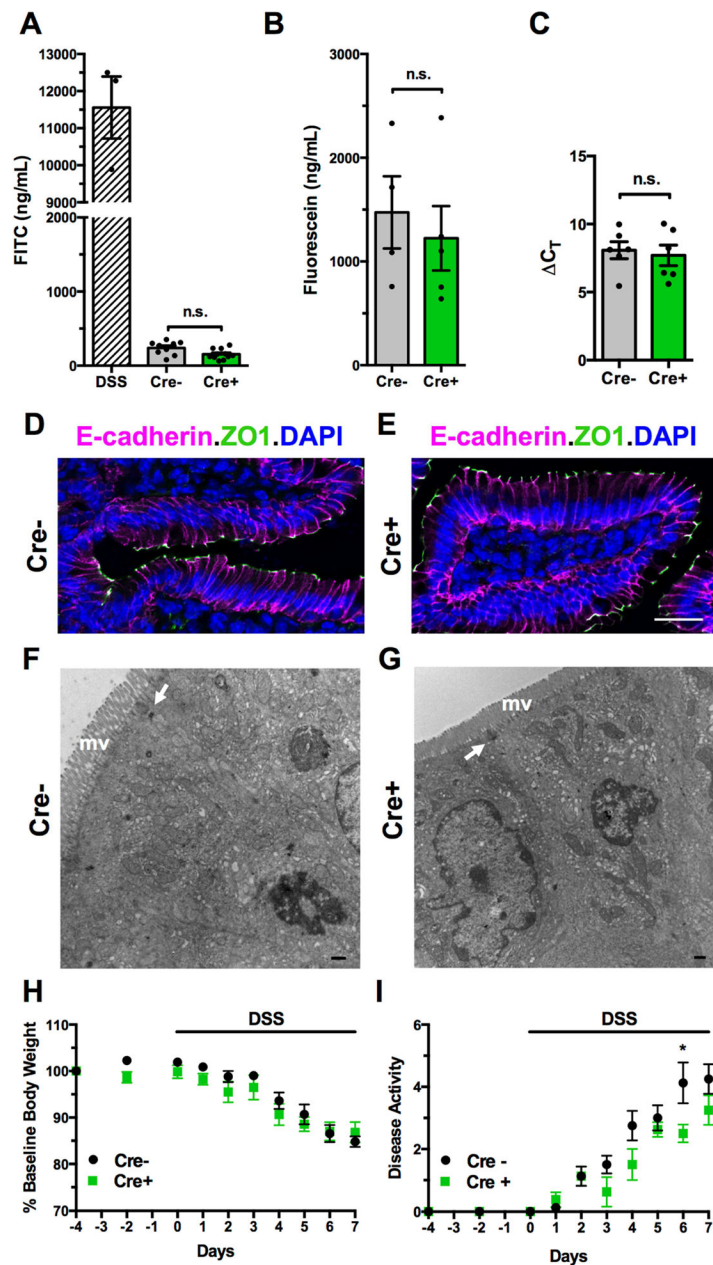Scale bars A, C, D, F=50μm; B, E=25μm; H–I=500nm; K–L=100μm.

Figure 2. Enteric glial elimination does not alter intestinal barrier permeability

(A) Serum FITC levels measured 4 hours after oral gavage of 4.4kDa FITC-dextran into Cre[−] and Cre[+] mice, suggests that glial elimination does not alter macromolecular intestinal permeability. Serum from wildtype mice treated with dextran sodium sulfate (DSS) was used as a positive control.

(B) Serum FITC levels measured 4 hours after oral gavage of a 478Da fluorescein conjugate into Cre[−] and Cre[+] mice. The smaller FITC-conjugate permeated the intestinal epithelium more than 4.4kDa FITC-dextran, yet again no difference was observed with glial elimination.

(C) Bacterial16S rRNA transcript levels in mesenteric lymph nodes were similar in Cre⁻ and Cre⁺ mice.

(D–E) Localization of E-cadherin and zonulin-1 (ZO1) immunoreactivities is not altered by glial elimination. DAPI marks nuclei. Scale bar=25μm.

(F–G) The ultrastructure of small intestinal enterocytes does not differ in Cre⁻ and Cre⁺ mice. Note the regular appearance of the microvillus border (mv), equivalent area of terminal web, intact junctional complexes (arrows), and non-vacuolated mitochondria in the cells of both animals. Scale bar=500nm.

(H–I) Cre⁻ and Cre⁺ mice with acute DSS colitis experience similar degrees of weight loss and disease activity. All mice received a single dose of tamoxifen at day -4 and then were exposed to 2.5% DSS in drinking water from day 0 onwards (n=4/group). * denotes $p < 0.05$ by 2-way ANOVA for Day 5 only. All other comparisons were not significant.
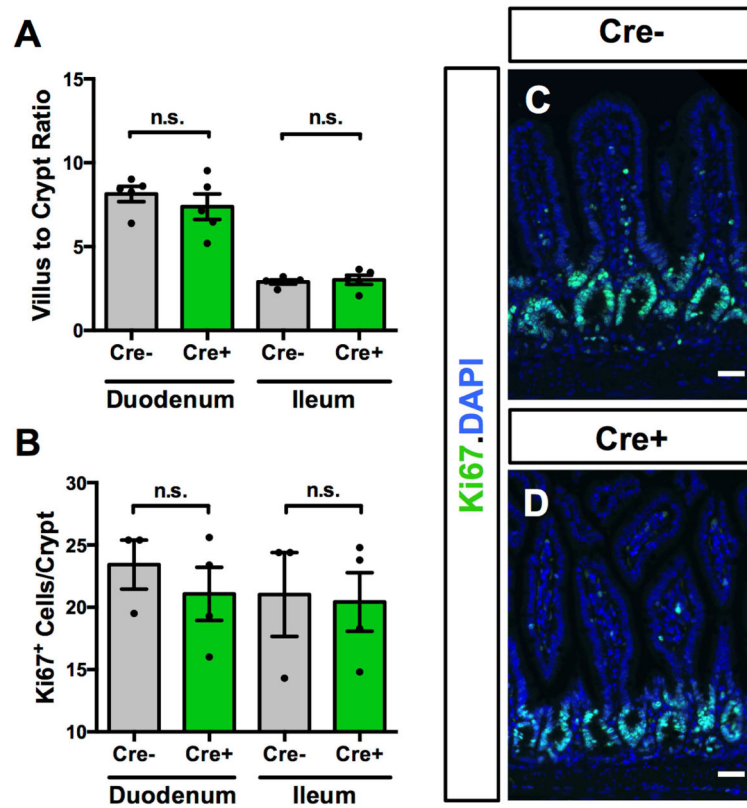
Figure 3. Enteric glial elimination does not alter epithelial proliferation

(A) Villus-to-crypt ratios in Cre⁻ and Cre⁺ mice were not different, and expected differences between ratios in duodenum and ileum were maintained.

(B–D) Ki67 immunoreactivity was not different in the epithelium of Cre⁻ and Cre⁺ mice. DAPI marks nuclei. Scale bar=50μm
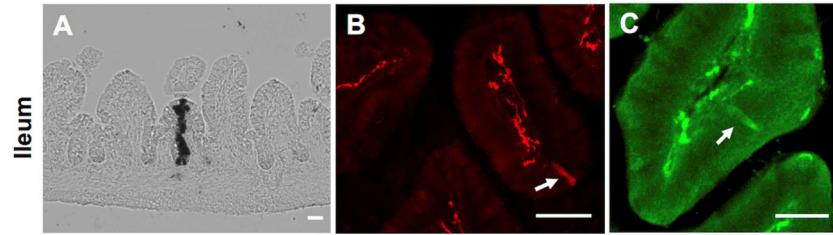
Figure 4. GFAP is expressed in rare epithelial cells in the distal small intestine

(A) X-Gal staining of ileum from Gfap^{CreER};Rosa26^{LacZ} mouse at 15dpt. A stripe of X-Gal labeled epithelial cells ranging from crypt base to villous tip in the small intestine suggests that Cre recombinase was expressed in an epithelial stem cell at the time of induction.

(B) TdTomato expression in the ileal mucosa of a 6-week-old GFAP^{Cre};Rosa26^{TdTomato} mouse. Arrow highlights TdTomato$^+$ epithelial cell.

(C) GFAP immunoreactivity in the ileal mucosa of a wild-type mouse. Arrow highlights GFAP-expressing epithelial cell.
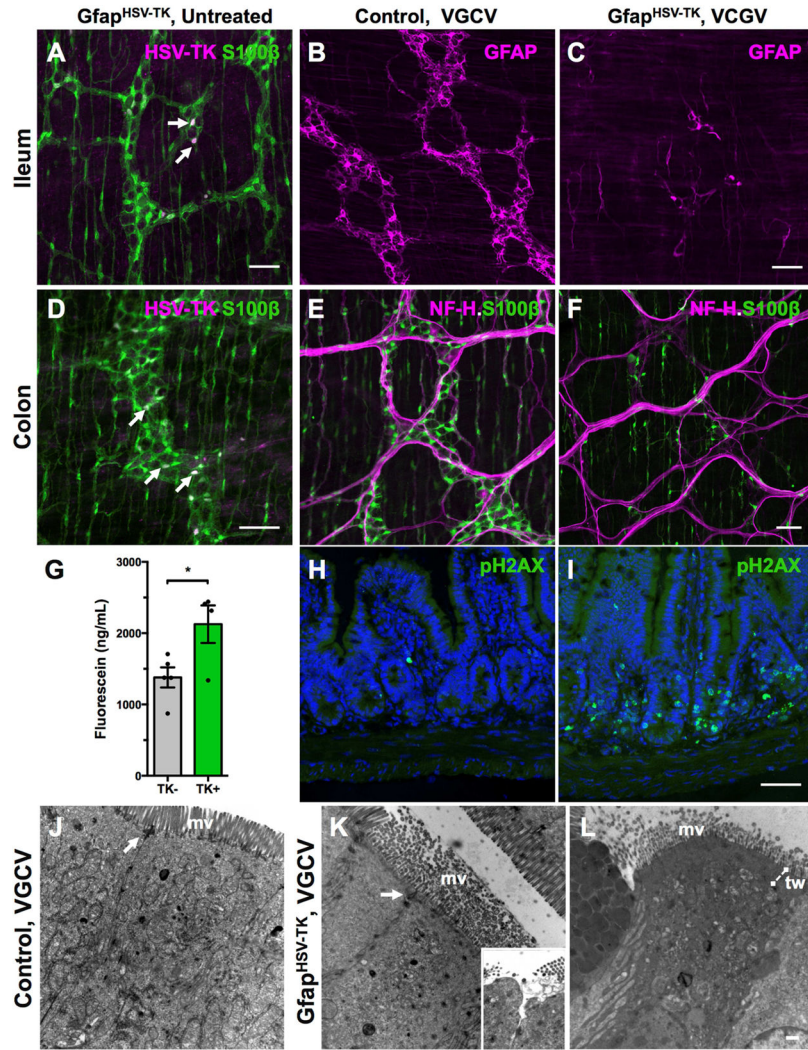
Scale bars A-B=50μm; C=25μm

Figure 5. Gfap^HSV-TK mice exhibit transgene expression throughout the intestine and signs of off-target injury to the epithelium

(A, D) Immunostaining for S100β and HSV-TK shows that only a subset of myenteric glia expresses TK (arrows) in the ileum and colon of untreated GFAP^HSV-TK mice. Intramuscular glia are S100β-immunoreactive but none express TK.

(B–F) Valganciclovir (VGCV) treatment eliminates both GFAP^+ (compare B and C) and S100β^+ (compare E and F) enteric glia in the small and large intestine of GFAP^HSV-TK mice. NF-H immunostaining highlights nerve fiber bundles in the myenteric plexus.

(G) Serum FITC levels 4 hours after oral gavage of 478Da fluorescein conjugate into TK^− and TK^+ mice treated with VGCV shows increased macromolecular intestinal permeability in TK^+ mice.

(H–I) Immunoreactivity of the apoptotic marker, phosphorylated histone 2AX (pH2AX) in cross-sections of ilea from TK^− (H) and TK^+ (I) mice treated with VGCV. Nuclei counterstained with DAPI. VGCV treatment increased apoptosis primarily in crypt cells, but also in the walls of the villi and in the lamina propria of the TK^+ mouse.

(J) The ultrastructure of enterocytes in the small intestine of a control TK$^-$ mouse treated with VGCV appears normal. The microvillus (mv) border was intact, and their core of actin filaments formed a terminal web and inserted into the adherens junction component of junctional complexes (arrow).

(K) The ultrastructure of some enterocytes in the small intestines of VGCV-treated TK$^+$ mice was abnormal. The microvilli were disorganized, lacked microfilament cores, and displayed variable diameters suggesting swelling. The area of the terminal web was expanded and appeared as an amorphous electron-dense mat. Occasionally, adherens junctions were disrupted although tight junctions remained intact. In rare cells, the underlying cytoplasm appeared to push the microvillus border aside and protrude into the lumen (see inset). In these regions, tight junctions were disrupted, opening a channel to the intercellular space.

(L) In an evident later stage of abnormality in a VGCV-treated TK$^+$ mouse, the cytoplasm of some enterocytes was electron dense, the terminal web (tw) region was expanded, and the mitochondrial matrix was vacuolated.
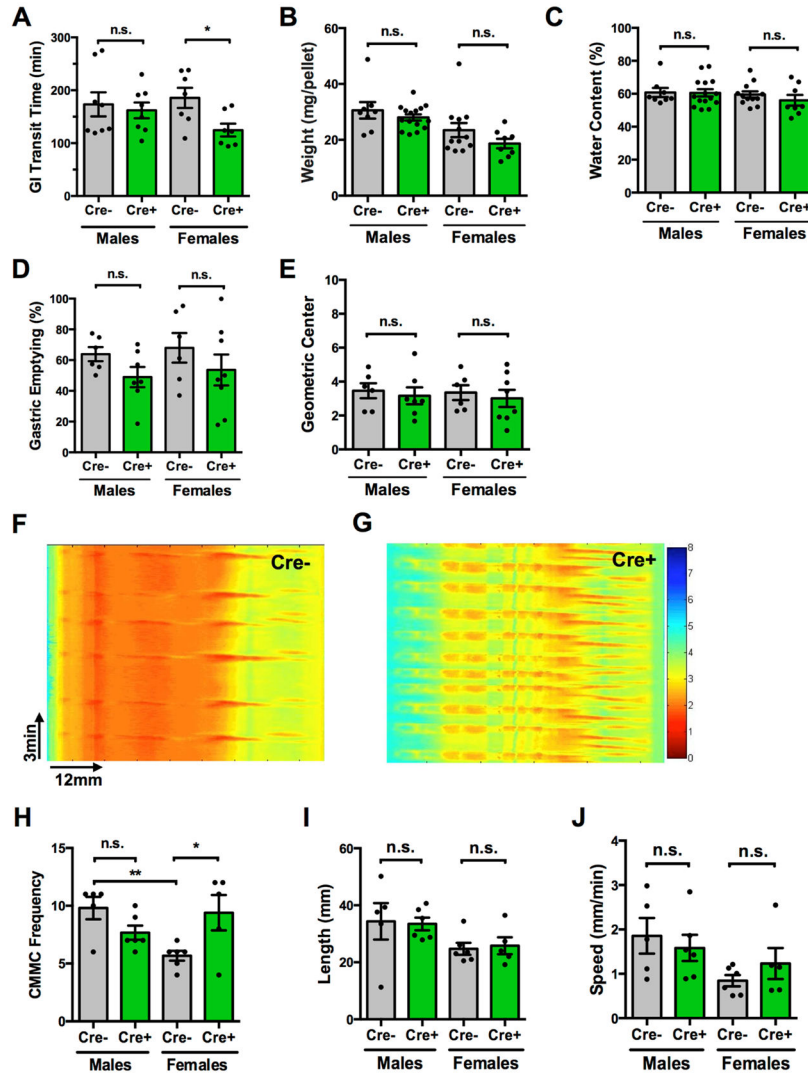
Scale bars A–I=50μm; J–L=500nm

Figure 6. Enteric glial elimination leads to a sex-dependent acceleration of colonic motility

(A) Total gastrointestinal transit time (GITT) in Rosa26[DTA] (Cre[−], control) and Plp1[CreER];Rosa26[DTA] (Cre[+], experimental) mice was compared at 5dpt. GITT was accelerated in female mice upon glial elimination, but was not altered in males.

(B–C) Stool mass per pellet and water content are unchanged by glial elimination in both sexes.

(D) Gastric emptying in male and female Cre[+] mice was no different than in Cre[−] littermates.

(E) Small intestinal transit time (geometric center is a proxy) was not affected by glial elimination in either sex.

(F–G) Spatiotemporal maps derived from video recordings of contractions of colons from Cre[−] (F) and Cre[+] (G) female mice. The maps show the patterns of contractions of the colonic musculature as a function of time. Colonic width is measured as a proxy for contraction, and is depicted on a colorimetric scale (red is maximal contraction). Colonic

migrating motor complexes (CMMCs) can be recognized as repeating proximal-distal contractions. X-axis = distance (mm), y-axis = time (min).

(H) In Cre⁻ controls, CMMC frequency was lower in females than males. Glial elimination increased female CMMC frequency to levels seen in males (n=5–6mice/group).

(I–J) Length and velocity of CMMC propagation are not different between Cre⁻ and Cre⁺ mice.
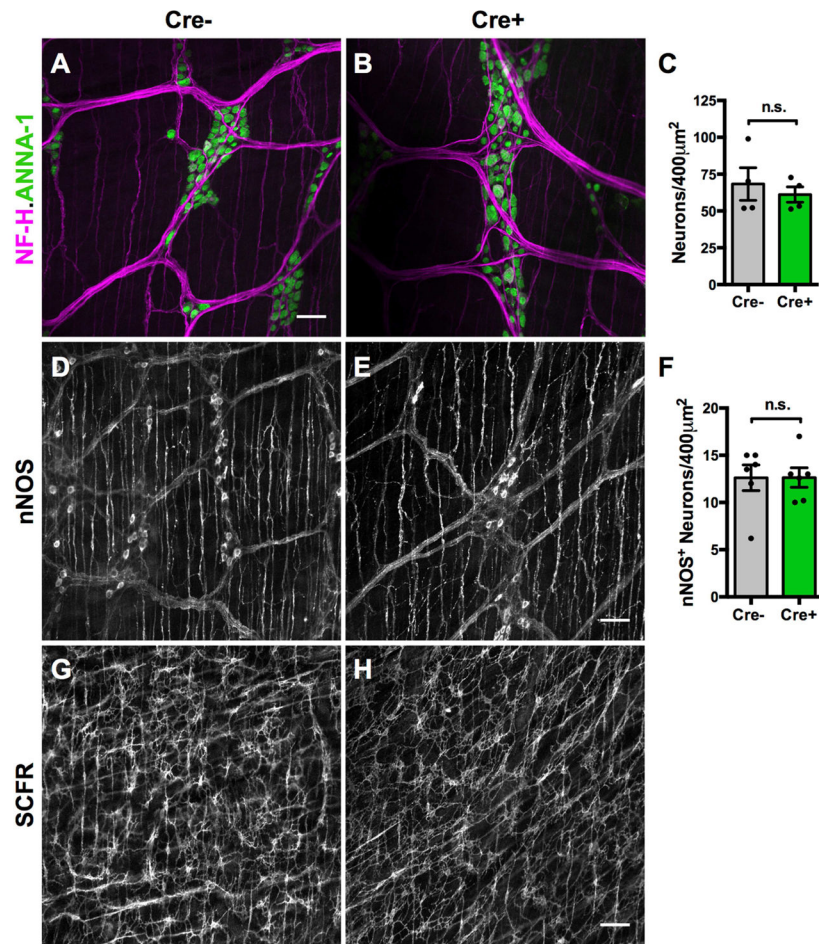
Figure 7. Enteric glial elimination does not alter neuronal survival or the myenteric networks of nitrergic neurons and interstitial cells of Cajal

(A–C) Immunoreactivities of neurofilament-H (NF-H) and the pan-neuronal marker, ANNA-1, in colons of Cre⁻ (A) and Cre⁺ (B) mice revealed no difference in myenteric neuron number or the pattern of interganglionic connectives.

(D–F) Neuronal nitric oxide synthase (nNOS) immunostaining revealed no difference in nitrergic neuron density in the myenteric plexus of Cre⁻ and Cre⁺ mice.

(G–H) SCFR immunoreactivity, a marker of interstitial cells of Cajal (ICC), revealed no difference in the ICC-MP network in Cre⁻ and Cre⁺ mice.

Scale bars A–B=25μm; D–G=50μm