Butyrate enemas enhance both cholinergic and nitrergic phenotype of myenteric neurons and neuromuscular transmission in newborn rat colon

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Preterm infants have immature GI motility patterns compared with term infants. In particular, duodenal clustered phasic contractions are more frequent, but the duration and amplitude of the clusters are smaller in preterm compared with term infants. In addition, the antrado- duodenal coordination is lower in preterm than in term infants (22). In the small intestine, clustered phasic contractions appear between 31 and 34 wk of gestation (WG), but only 50% of them are propagated aborally. With term, these clusters increase in duration and frequency, and a larger proportion of them are aborally propagated (4). In the colon of term infants, propulsive motor activity occurs only 24–48 h after birth, leading to meconium expulsion (3). However, the delay until the beginning of meconium expulsion and the overall duration of the meconium expulsion is larger in preterm than in term infants (3). Altogether, the whole gut transit time is increased in preterm compared with term infants (28). This reduced GI motor activity is then responsible for gastric stasis (36) and prolonged ileus (39) and is also probably involved in the pathogenesis of digestive complications observed in preterm infants such as necrotizing enterocolitis (26) and spontaneous intestinal perforation (5). In addition, severe GI motility dysfunctions observed in populations of preterm infants can prevent enteral feeding, leading to a prolonged parenteral nutrition and associated morbidity (16, 40). Therefore, approaches aiming at enhancing the maturation of GI functions, in particular colonic motility, could be of major therapeutic interest.

Among the key regulators of the GI motility is the enteric nervous system (ENS) (38). The ENS is a neuronal network organized in two major plexus locations (myenteric and submucosal plexus) located all along the gut. Myenteric neurons control GI motility and in particular peristalsis (25). Excitatory myenteric motoneurons contain acetylcholine and substance P that induce smooth muscle contraction, whereas inhibitory motoneurons contain nitric oxide (NO) and vasointestinal peptide, which induce muscle relaxation (15). Although the GI tract is fully colonized by the ENS during embryogenesis [embryonic day 9–15 in mice (9, 21), WG 4–7 in humans (13, 45)], increasing data suggest that maturation of the ENS still occurs after this colonization, even after birth (20). In particular, in rat neonates, the proportion of nitrergic myenteric neurons increases early in the postnatal life followed by a significant increase in the proportion of cholinergic neurons (44). In the human and guinea pig small intestine, a stronger nitrergic inhibitory component of the neuromuscular transmission is observed in neonates than in adults (32, 46). The maturation of the cholinergic phenotype of myenteric neurons is correlated with the development of colonic migrating motor complexes in mice (37) and colonic propulsive motility in rats...
(44). However, environmental factors putatively involved in the postnatal maturation of the ENS and colonic motility remain unknown.

Among such factors, nutrients or nutrient-derived products such as short-chain fatty acids (SCFAs) could play a critical role. SCFAs are metabolites resulting from bacterial fermentation of resistant starch (43). Concentrations of SCFAs in the colon increase both in humans and rats after birth (2, 31). Fecal concentrations of SCFAs are 3.2-fold lower in infants born before 33 WG compared with infants born after (12). In rat pups, butyrate concentration in ceco-colonic content increases from 3 to 33 μmol/g between postnatal day (PND) 18 and PND 40 (2). A recent study has shown that butyrate increases the proportion of cholinergic myenteric neurons associated with an increased colonic motility in adult rats (41). However, whether butyrate also has similar phenotypical and functional effects in rat pups remains currently unknown.

Therefore, the aim of this study was to determine the effects of administration of butyrate enemas in the postnatal period on the neurochemical phenotype of ENS and its functional impact on in vivo and ex vivo motility.

MATERIAL AND METHODS

Animal Models

Experiments were carried out in accordance with the recommendations and approval of the local Animal Care and Use Committee of Nantes (France). Primiparous timed-pregnant Sprague-Dawley female rats (Janvier Laboratories, Le Genest Saint Isle, France) were obtained at 13–14 days of gestation. Rats were accustomed to laboratory conditions for 1 wk before delivery and individually housed in cages on a 12-h:12-h light/dark cycle with free access to water and food (UAR, Epinay-sur-orge, France). Mothers and their pups (10–16 per litter) were isolated in the same conditions during the whole experiment. Day of birth was considered to be PND 0.

Administration of Enemas

Enemas were performed daily in rats between PND 7 to PND 17. A 24-gauge cannula (Fine Science Tools, Vancouver, Canada) was inserted 5 mm in the colon from the rectum to the cecum (Fig. 1). Methylene blue along the colon following an enema (20 μl/g) was performed in a 17- day-old postnatal rat (A). Methylene blue stained the entire colon from the rectum to the cecum (B).

Evaluation of Colonic Motility

In vivo experiments. BEAD LATENCY. Distal colon transit time was measured at PND 21 using a method previously described in rats (44). A 2-mm-diameter glass bead (Sigma) was inserted 5 mm in the colon using a 5-French-gauge-polished-end urethral catheter (Porgès, Le Plessis Robinson, France). After bead insertion, pups were isolated in individual cages. Distal colonic transit time (bead latency) was determined by measuring the time required for the expulsion of the bead.

WATER CONTENT OF FECES. Pups were isolated in individual cages without access to food and water for 1 h at PND 21. Feces were collected and weighed. Feces were then dried at 50°C for 1 wk and weighed. The water content of feces was evaluated and expressed as a percentage of wet weight.

Ex vivo experiments. Ex vivo neuromuscular transmission was evaluated as previously described (44). At PND 21, rats were killed by cervical dislocation, and the colon was removed (except the distal extremity ~5 mm from anus). The colon was placed in cold oxygenated (5% CO2-95% O2) Krebs solution containing (in mM) 117.0 NaCl, 4.7 KCl, 1.2 MgCl2, 1.2 NaH2 PO4, 25.0 NaHCO3, 2.5 CaCl2, and 11.0 glucose.

Segments of distal colon were placed in the longitudinal direction in a 7-ml organ bath containing oxygenated Krebs solution (37°C) and were stretched with a preload of 0.04 to 0.06 mN of tension. Preparations were equilibrated for 60 min. Isometric contractions were recorded with force transducers (no. 7005; Basile, Comerio, Italy) and data acquired onto a PowerMac Performa 7100/80 computer equipped with the MacLab/4s system (ADI, Bremen, Germany). Activation of enteric neurons was performed by electrical field stimulation (EFS) using a stimulator connected to two platinum ring electrodes (10 V, duration of pulse train: 10 s; pulse duration: 300 μs; frequency: 30 Hz). This procedure was repeated three times with 10-min periods between stimulations. The response of colonic longitudinal muscle to EFS was also measured in the presence of NO synthase (NOS)
acetylcholinesterase assay kit, Invitrogen) and normalized to the number of neurons per ganglia and the percentage of ChAT-IR or nNOS-IR neurons normalized to the total number of Hu-IR neurons. Structures not clearly identified as ganglia were not analyzed. Data are expressed as the means ± SE. The significance of differences was determined using Mann-Whitney U-test to compare two means, Wilcoxon matched-pairs test for paired data, or Kruskal-Wallis test to compare more than two groups, followed by Dunn’s multiple-comparison test. The weight gain during the time of experiments was determined by using a linear regression fit. Differences were considered statistically significant for P < 0.05.

RESULTS

Impact of Butyrate Enemas On Bead Latency Time

In a first part of the study, we characterized the impact of butyrate enemas on the distal colonic motility. We first showed that 2.5 mM butyrate significantly reduced the bead latency time compared with control (sham enemas) (P < 0.001) (Fig. 2A). In addition, enemas of 5 mM butyrate also significantly reduced the bead latency time (P < 0.001) (Fig. 2A). However, bead latency time was not modified after saline, mannitol, or sham enema compared with unmanipulated animals (Fig. 2B). We next used 2.5 mM butyrate enemas for the remaining sets of experiments, and for controls sham enemas were used.

Impact of Butyrate Enemas On Morphological Parameters

During the period of butyrate enema administration, the weight gain over the time was not significantly different from controls.
from controls (Fig. 3A). At PND 21, the colon length was similar between controls (8.9 ± 0.5 cm; n = 5) and butyrate-treated animals (8.7 ± 0.2 cm; n = 6). Butyrate enemas did not modify morphological parameters such as the height of the mucosa, the submucosa, the crypts, the circular or longitudinal muscle layers compared with controls (Fig. 3B).

**Impact of Butyrate Enemas On the Neurochemical Phenotype of Enteric Neurons**

We next sought to determine the impact of butyrate on the neurochemical phenotype of enteric neurons of the distal and proximal colon.

**Distal colon.** In the myenteric plexus, butyrate enemas did not modify the number Hu-IR cells per ganglia (Fig. 4, B and C) compared with controls (Fig. 4, A and C). However, butyrate significantly increased the proportion of both ChAT-IR neurons (43%; n = 8; P = 0.02) (Fig. 4, E and F) and nNOS-IR neurons (27%; n = 8; P = 0.04) (Fig. 4, H and I) compared with controls (Figs. 4, D, F, G, and I, respectively). This increase in the proportion of ChAT-IR neurons was associated with a significant increase in the acetylcholine amount in the distal colon of butyrate-treated animals (10.1 ± 3.3 μmol/μg of NSE; n = 6) compared with controls (2.0 ± 0.8 μmol/μg of NSE; n = 6; P = 0.03). Saline enemas did not modify the number of Hu-IR cells per ganglia compared with controls (Fig. 4, C and G).
ganglia compared with controls (31.9 ± 0.8 cells; n = 4 vs. 32.2 ± 0.9 cells; n = 8, respectively) or the proportion of ChAT-IR and nNOS-IR neurons compared with controls (15.3 ± 2.8%; n = 4 vs. 17.2 ± 1.0%; n = 8 and 20.6 ± 1.9%; n = 4 vs. 19.7 ± 1.4%; n = 8, respectively).

Similarly, mannitol enemas did not modify the number of Hu-IR cells per ganglia compared with controls (31.1 ± 0.7% cells; n = 4 vs. 32.2 ± 0.9% cells; n = 8, respectively) or the proportion of ChAT-IR and nNOS-IR neurons compared with controls (13.0 ± 1.7%; n = 4 vs. 17.2 ± 1.0%; n = 8 and 19.5 ± 0.9%; n = 4 vs. 19.7 ± 1.4%; n = 8, respectively).

We next determined whether butyrate enemas could modify the phenotype of submucosal neurons in the distal colon. Butyrate did not modify the number of Hu-IR cells per ganglia (8.6 ± 1.8 cells; n = 5) compared with controls (9.7 ± 3.2 cells; n = 4). However, butyrate increased the proportion of nNOS-IR neurons (26.2 ± 3.5%; n = 5) compared with controls (12.7 ± 1.6%; n = 4; P = 0.03) and tended to increase the proportion of ChAT-IR neurons (43.3 ± 3.9%; n = 5) compared with controls (35.1 ± 1.8%; n = 4; P = 0.1).

Proximal colon. We next determined whether butyrate enemas could also impact the neurochemical phenotype of myenteric neurons in the proximal colon.

The number of Hu-IR cells per myenteric ganglia was not different between controls and butyrate-treated animals (35.1 ± 1.0 cells; n = 5 vs. 36.4 ± 0.8 cells; n = 6, respectively). In contrast to distal colon, butyrate did not modify the proportion of ChAT-IR neurons (26.2 ± 1.5%; n = 6) compared with controls (25.7 ± 0.5%; n = 5). Butyrate also did not modify the proportion of nNOS-IR neurons (22.2 ± 1.7%; n = 6) compared with controls (22.4 ± 0.7%; n = 5).

We next compared the phenotype of myenteric neurons between the proximal and distal colon. In control animals, the number of Hu-IR cells/ganglia tended to be higher in the proximal compared with the distal colon (35.4 ± 1.0 cells vs. 32.2 ± 0.9 cells, respectively; n = 5; P = 0.09). The proportion of ChAT-IR neurons was significantly larger in the proximal than in the distal colon (25.4 ± 0.5% vs. 14.6 ± 2.2%, respectively; n = 5; P = 0.02). The proportion of NOS-IR neurons tended to be larger in the proximal than in the distal colon (22.4 ± 0.7% vs. 20.3 ± 1.7%, respectively; n = 5; P = 0.06). In butyrate-treated animals, the number of Hu-IR cells/ganglia was higher in the proximal colon compared with the distal colon (36.4 ± 0.8 vs. 31.5 ± 1.0, respectively; n = 5; P = 0.04). The proportion of ChAT-IR neurons remained larger in the proximal than in the distal colon (26.3 ± 1.2% vs. 20.3 ± 2.0%, respectively; n = 5; P = 0.02), and the proportion of NOS-IR neurons tended to be larger in the distal than in the proximal colon (22.2 ± 1.7% vs. 27.0 ± 2.2%, respectively; n = 5; P = 0.09).

Impact Of Butyrate On The Distal Colonic Permeability

Butyrate enemas could also impact the paracellular permeability in the distal colon. Sulfonic acid flux across the colonic mucosa was identical in butyrate-treated animals compared with controls (Fig. 6A). Butyrate enemas did not modify the proportion of water in feces (44.6 ± 5.7%; n = 5) compared with control (47.8 ± 2.6%; n = 4) (Fig. 6B).

Discussion

This study showed that butyrate enemas induce in rat pups profound neuroplastic changes in myenteric and submucosal neurons characterized by an increase in the proportion of both cholinergic and nitrergic neurons. Furthermore, this neuroplasticity was associated with an enhanced distal colonic motility and changes in neuromuscular transmission.

A first finding of our study was the ability of enemas to target the ENS in the colon. Indeed, in preliminary results of this study, we showed that a substance administered by enemas was able to reach the entire colon. For the colon, enemas represent various advantages over the oral route such as to bypass the intestinal assimilation of delivered substances and prevent putative deleterious effects of digestive secretory products (acid, biliary salts, or pancreatic enzymes) on the administered substance. Specific targeting of the colon can be of particular interest in the context of postnatal motility disorders of the lower GI tract. Indeed, enemas are used in preterm infants with a delayed meconium expulsion. These approaches attempt to avoid prolonged ileus complications such as intestinal perforation, necrotizing enterocolitis, and parenteral support morbidity. The therapeutic aim of these enemas is in general to decrease meconium viscosity with saline solution.
(NaCl 0.9%) (11, 19) or with osmotic agents such as Gastrografin (16) or iopamidol (29). Such approaches have been associated with complications like dehydration (34), intestinal perforation, or rectal injury (1, 7, 10, 18). However, it remains unclear whether these complications result from the procedure by itself or are a primary consequence of colon dysfunctions due to immaturity. In our study, we did not observe any rectal bleedings, intestinal perforations, or peritonitis as consequences of enema procedures. In addition, our data suggest that butyrate enema-induced functional changes were not attributable to changes either in distension induced by enemas or by osmolarity because enemas of saline and mannitol, respectively, did not modify distal colonic transit, nor did they modify the neurochemical phenotype of myenteric neurons. Consistently, in preterm infants, saline enemas did not improve colonic motility (19). Furthermore, we showed in our study that butyrate enemas (2.5 mM) did not impact colonic permeability. This result is consistent with a previous ex vivo study.

Fig. 5. Impact of butyrate enemas on neuromuscular transmission assessed ex vivo. Distal colonic longitudinal muscle segments were stimulated by electrical field stimulation (EFS). The area under the curve (AUC) of EFS-induced contractile response was analyzed in absence or in presence of N-nitro-l-arginine methyl ester (l-NAME) or atropine (A). The EFS-induced AUC was larger in butyrate-treated animals compared with controls (sham enemas) (B) (#P < 0.05; Mann Whitney U-test; n = 6). In presence of l-NAME, EFS-induced AUC was significantly increased in both groups (B) (*P < 0.05, Wilcoxon matched-pairs test; n = 6) and was also larger in butyrate-treated animals compared with controls (sham enemas) (B) (#P < 0.05; Mann Whitney U-test; n = 6). However, the amplitude of l-NAME-sensitive AUC was larger after butyrate treatment compared with controls (sham enemas) (C) (*P < 0.05; Mann Whitney U-test; n = 6). In the presence of atropine, EFS-induced AUC was significantly decreased only in the butyrate-treated group (B) (*P < 0.05, Wilcoxon matched-pairs test; n = 6). Furthermore, the amplitude of atropine-sensitive AUC was higher in the butyrate group compared with control (sham enemas) (D) (*P < 0.05; Mann Whitney U-test; n = 6).
showing that low doses of butyrate (less than 10 mM) did not impact permeability, whereas higher doses of butyrate (more than 10 mM) reduced paracellular permeability (27).

A major finding of our study was the demonstration that butyrate enemas were able to induce neuroplastic and functional changes in the ENS of distal colon of rat pups. In particular, the butyrate-induced increase in the proportion of cholinergic myenteric neurons and cholinergic neuromuscular transmission was reminiscent to the effects of butyrate observed in the adult rat with in vivo cecal infusion of butyrate (41). However, whether these neuroplastic changes induced by butyrate are maintained over time remains currently unknown. In adult rats, butyrate effects were observed in the proximal colon with a concentration of 5 mM and were absent with 1 mM of butyrate. We observed an effect at a lower concentration of butyrate in our study, i.e., 2.5 mM. This concentration of butyrate is in the range of the one observed during the postnatal period in rat pups. Indeed, concentration of butyrate measured in ceco-colonic content is 3 µmol/g at P18 and reaches 33 µmol/g of dried feces at P40 (2). We observed an effect of butyrate enemas on the neurochemical phenotype of ENS only in the distal colon and not in the proximal colon although enemas were able to reach the cecum. Although an absence of effect could be attributable to lower time of exposure of the proximal colon than distal one to butyrate, one cannot exclude also that region-specific differences in sensitivity to butyrate (i.e., proximal vs. distal colon) could exist. Indeed, butyrate concentrations are higher in the cecum compared with the rectum (8). Conversely, the expression of the monocarboxylate transporter (MCT)-1 (expressed on colonocytes and transporting butyrate) increases along the colon and is maximum in the distal segment (17). Whether similar regional or age-dependent changes occur with other monocarboxylate transporters such as MCT-2, which is expressed on myenteric neurons (41), remains currently unknown. However, we showed similar expression of MCT-2 both in distal and proximal colon (data not shown).

Surprisingly, we also observed a major effect of butyrate on the nitrergic phenotype in newborn rats, whereas in adult rats butyrate had no effect on this subtype of neurons (41). Mechanisms responsible for these age-dependent differences remain unknown but could be attributable to differences in the epigenetic state of myenteric neurons during the postnatal period compared with adulthood. This could be highly likely because butyrate effects on ENS phenotype were shown to be associated with epigenetic changes in neurons (45). These effects of butyrate could also be attributable to an increase in the proliferation of neuronal progenitors, which are still present in the postnatal period (24). In the brain, butyrate administration increases bromodeoxyuridine incorporation of progenitor cells in the subventricular zone after ischemia (23). However, such a mechanism might not be involved in our study, as the number of neurons per ganglia remained unchanged after butyrate treatment.

Another interesting finding of this study was that the proportion of nitrergic and cholinergic neurons followed an aboral gradient between the proximal and distal colon. Such a gradient has been already described for the nitrergic neurons in adult rats (42) and guinea pigs (14) but not for cholinergic neurons. This suggests that, during the postnatal period, a rostro-caudal gradient in the maturation of the neurochemical phenotype exists, which could be reminiscent of the gut colonization by neural crest stem cells during embryogenesis.

In conclusion, we have shown that butyrate enemas enhance colonic motility in rat pups by modulating the neurochemical phenotype of myenteric neurons. This study could serve as a basis for the development of therapeutic approaches in preterm infants with colonic motility disorders characterized by reduced transit time.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: E.S., P.d.V., R.S., and M.N. conception and design of research; E.S., R.S., and F.C. performed experiments; E.S. analyzed data; E.S. and M.N. interpreted results of experiments; E.S. prepared Figs.; E.S. and M.N. drafted manuscript; E.S., P.d.V., R.S., F.C., and M.N. edited and revised manuscript; E.S. and M.N. approved final version of manuscript.
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