Postnatal development of the myenteric glial network and its modulation by butyrate

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1INSERM, U913, Nantes, France; 2Université Nantes, Nantes, France; 3CHU Nantes, Hôtel Dieu, Institut des Maladies de l’Appareil Digestif, France; and 4Centre de Recherche en Nutrition Humaine, Nantes, France

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Cossais F, Durand T, Chevalier J, Boudaud M, Kermarrec L, Aubert P, Neveu I, Naveilhan P, Neunlist M. Postnatal development of the myenteric glial network and its modulation by butyrate. Am J Physiol Gastrointest Liver Physiol 310: G941–G951, 2016. First published April 7, 2016; doi:10.1152/ajpgi.00232.2015.—The postnatal period is a critical time window during which major adaptive processes occur. Shortly after birth, the gut undergoes various physiological changes that are important for the acquisition of a functional gastrointestinal (GI) tract that guarantees optimal absorption of nutrients, efficient protection against pathogens, and good transit for food and secretions. These adaptive processes include modifications of intestinal permeability (14, 30) but also changes in motility patterns. Indeed, the first GI motility patterns in fetal mice do not require enteric neurons for their generation, and neuronal control of the small intestine begins shortly before birth (14, 14a, 33). Changes in intestinal motility are associated with the development and maturation of the enteric nervous system (ENS), and we recently showed that the postnatal development of intestinal motility in rats is related to the concomitant increase in the proportion of enteric cholinergic neurons (14a, 34). Whereas neuronal genesis and maturation in the GI tract have been quite well described, little is known about the postnatal development of enteric glial cells (EGC). These cells are present in the myenteric and submucosal ganglia, along the nerve fibers that innervate the gut, and in all the layers of the gut wall including muscle and epithelium (28). Like central astrocytes, they have an important role in modulating the neuronal circuitry (15, 28), and they express glial markers such as glial fibrillary acid protein (GFAP) and the calcium-binding protein S100β. Evidence collected during the past 10 yr suggest a larger role for EGC in the GI tract. This includes neuroprotection (12), modulation of the enteric neuronal phenotype (1), regulation of intestinal motility (5, 23), direct control of epithelial cell properties, and regulation of intestinal epithelial barrier functions (2, 27). These pleiotropic functions have been suggested to contribute to ENS and gut homeostasis (28).

Like enteric neurons, EGC arise from neural crest cell-derived progenitors that have entirely colonized the gut by embryonic day 14 (E14) in mice and by E16.5 in rats (39). Differentiation of enteric neurons usually precedes differentiation of EGC with an expression of the panneuronal protein, the ELAV-like RNA-binding protein, HuC/D, starting at E12.5, whereas glial markers such as S100β appear at E14.5, and GFAP appears at E16.5 in the mouse duodenum (38). To define the subsequent events leading to the formation of mature glial network in the intestine, we decided to analyze the postnatal evolution of the myenteric glial network in the colon of rat pups. We also investigated the effect of extrinsic factors on the proliferation and differentiation of EGC because recent data suggested that environmental factors affect postnatal development of the ENS (11, 14, 36). Among those suggested to have a critical role are luminal factors endogenously produced in the colon, such as microbiota-derived short-chain fatty acids (SCFA). Resulting from the bacterial fermentation of dietary fiber and resistant starch, their concentration after birth progressively increases in the colon in humans and rats (4, 29). In particular, we focused our work on the effect of butyrate because this SCFA was shown to enhance the cholinergic phenotype of myenteric neurons and related neuromuscular functions (35, 36).

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 were individually housed in cages on a 12:12-h light-dark cycle with free access to water and food (UAR, Épinay-sur-Orge, France). Mothers and their pups (10–16 per litter) were kept in the same conditions during the all experiments.
Day of birth was considered to be postnatal day 0 (P0). Rat pups aged from P1 to P14 were euthanized by decapitation and by cervical dislocation at later stages.

Administration of Butyrate Enemas

Enemas of saline (NaCl 0.9%, control) and sodium butyrate (2.5 mM; Sigma, Saint Quentin Fallavier, France) were performed daily in rat pups between 5 and 21 days of life as described earlier. For short-term study, enemas of saline (NaCl 0.9%, control) and sodium butyrate (2.5, 10, and 40 mM) were performed at P6. Briefly, a 24-gauge cannula (Fine Science Tools, Vancouver, BC, Canada) was introduced in the rectum up to 5 mm, and a volume of 20 μl of animal weight was injected. Enemas were injected slowly (10 s per enema), the cannula was then removed, and pups were maintained muzzle down for 20 s.

Enteric Glial Cell Line

The rat EGC cell line JUG-2 was used in the present study (2, 37). JUG2 cells were obtained from an ENS primary culture derived from rat embryonic intestine (10). Briefly, after 13 days of culture, embryonic primary cultures of ENS were trypsinized and seeded in serum-containing medium after differential centrifugation. Following 7 days of culture, isolated areas of cells that exhibited morphology of glial cells were trypsinized using a cloning cylinder and seeded in a culture flask in serum-containing medium. After 1 mo in culture, cells were immunoreactive for glial but not for neuronal or myofibroblast markers. JUG2 cells were cultured in DMEM (4.5 g/l glucose; Life Technologies, Cergy-Pontoise, France) supplemented with 10% heat-inactivated FCS (Biowest, Nuaille, France), 50 IU/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). EGC were seeded at a density of 5,000 cells per well in 96-well plates (Corning, Avon, France). The day after seeding, butyrate, acetate, or propionate (500 μM; Sigma) or trichostatin (TSA, 0.25 μM; Sigma) was added to the culture medium and EGC were cultured for 7 days (the time at which control cells usually reached confluence).

EGC Primary Cultures

EGC primary cultures were derived from the colons of rats aged P14 and P36. Briefly, longitudinal muscle/myenteric plexus associated with circular muscle layer were obtained with a scalpel and removed from the underlying mucosa with a pair of fine dissection forceps. The specimens were collected in GentleMACS tubes C (Miltenyi Biotec, Paris, France) containing 5 ml of a complete DMEM/F12 medium consisting of DMEM/F12 (1:1) supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1, 1 μg/ml amphotericin B, 20 μg/ml gentamicin, 6 mM glutamine, and 2.1 g/l NaHCO3 (all from Life Technologies). Protease (250 μl) (stock solution 5 g/l), 250 μl collagenase (stock solution 20 g/l), and 400 μl BSA (stock solution 50 g/l) (Sigma) were added to each tube. For EGC at P14, the tubes were incubated under gentle agitation on a rocker at 37°C for 1 h without additional mechanical dissociation. For EGC at P36, the tubes were placed in a GentleMACS dissociator (Miltenyi Biotec), and a mechanical dissociation procedure was performed (program m-brain 01-02). Next, the tubes were incubated under agitation on a rocker at 37°C for 20 min, and an additional dissociation run was performed. For cells in both the P14 and P36 groups, 10 ml of DMEM/F12 was added to the preparations before centrifugation at 1,500 revolutions per minute for 5 min. Pellets were washed once with ice-cold sterile Krebs solution and, after centrifugation, were resuspended in 10 ml of DMEM/F12 complete medium. The suspension was then transferred in Petri dishes (Ø 10 cm). For isolation of ganglia and interganglionic strands, Petri dishes were placed under an inverted microscope and individual ganglia were picked up using a pipette fitted with a 200-μl sterile tip. Ten to 20 ganglionic structures were then seeded in gelatin-coated (0.5% in PBS; Sigma) 24-well plates and cultured in DMEM/F12 complete medium for 72 h at 37°C in a 5% CO2 incubator. The medium was then replaced with complete DMEM medium (DMEM 4.5 g/l glucose supplemented with 50% FCS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin). EGC were maintained in culture for 35–60 days, until confluence was reached. Purity of the culture was assessed by immunohistochemistry. Cultures that were contaminated with more than 20% of cells that were positive for α-smooth muscle actin were discarded and not included in the study.

Cell Proliferation Assay

EGC were harvested with 1% trypsin-EDTA (Life Technologies) and homogenized. Cells were counted in a blinded fashion using Malassez slides (VWR International, Strasbourg, France) and fluorescence-activated cell sorting (FACS) (BD Bioscience, Erembourg, Belgium). Cell death was estimated using trypan blue assay and 7-aminoactinomycin D (BD Biosciences) incorporation using FACS analysis.

Cell Cycle Assay

JUG-2 cells were plated in a 24-well plate at 50,000 cells per well and cultivated in complete DMEM medium for 24 h. Cells were then treated (or not treated) with 500 μM butyrate or vehicle for 48 h. Medium was then removed and, after one wash with PBS, 30 μl of trypsin was added per well. After 90 s of incubation at 37°C, the trypsin reaction was stopped by adding 160 μl of medium containing 10% FCS per well, and the cells were transferred into a 96-well conical-bottomed plate. Plates were centrifuged to remove medium and, after one wash with PBS supplemented with 2% FCS and 2 mM EDTA, 100 μl of prewarmed PBS supplemented with 0.1% saponin was added per well for soft permeabilization on ice for 5 min. Then plates were centrifuged to remove PBS, and a mixture (100 μl per well) was added consisting of PBS supplemented with 20 μg/ml ribonuclease A from bovine pancreas (Sigma-Aldrich) and 10 μg/ml propidium iodide (PI). Thirty minutes after PI treatment, fluorescence was measured with an LSRII cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Immunofluorescence Staining

Cell cultures. Cells were fixed for 30 min at room temperature (RT) in 0.1 M PBS containing 4% paraformaldehyde (4% PFA), then washed three times in PBS before immunohistochemical staining.

Tissue preparation. Segments of proximal (1 cm adjacent to the cecum) and distal (1 cm, directly above the segment used in motility studies) colons were opened along the mesentery, pinned in a Sylgard-coated (Dow Corning, Midland, MI) Petri dish, and fixed for 3 h at RT in 4% PFA. The whole mounts of myenteric plexus obtained from the microdissection of mucosa and circular muscle were first permeabilized for 3 h at RT with PBS containing 0.1% sodium azide, 10% donkey serum, and 0.5% Triton X-100. Immunostaining was performed by incubation of the specimens with primary antibodies for 16 h at 4°C, followed by three washings in PBS. The nuclei were stained using DAPI (Sigma), and specimens were then incubated with the secondary antibodies for 1 h. Primary antibodies included goat anti-Sox10 (1:200, sc-1732, Santa Cruz Biotechnology), mouse anti-Hu/C/D (1:200, A21275; Life Technologies), rabbit anti-GFAP (1: 1,000, Z0334; Dakocytomation, Glostrup, Denmark), rabbit anti-S100β (1:10,000, Z0311; Dakocytomation), mouse anti-S100β (1: 500, Ab11178; Abcam, Cambridge, UK), rabbit anti-Ki67 (1:500, Ab66155; Abcam), rabbit anti-caspase 3 (1:500, C8487; Sigma), and rabbit anti-acetyl H3k9 (1:1,000, Ab10812; Abcam). Secondary antibodies included anti-mouse carboxymethylindocyanine (Cy3) (1:500, Jackson ImmunoResearch, Suffolk, UK), anti-goat Cy3 (1:500, Jackson ImmunoResearch), and anti-rabbit FP488 (1:200, Interchim, Montluçon, France), and anti-mouse CY5 (1:500, Jackson ImmunoResearch). Specimens were viewed under an Olympus IX 50 fluores...
cDNA was synthesized using SuperScript III Reverse Transcriptase (Life Technologies, Saint-Aubin, France) according to the manufacturer’s recommendations. Real-time quantitative PCR (qPCR) was performed. Amplification reactions were performed using the ABsolute Blue SYBR green fluorescent kit (Applied Biosystems, Foster City, CA) or performed using a FastSybr green mastermix kit (Applied Biosystems, Foster City, CA) or performed using a FastSybr green mastermix kit (Applied Biosystems) and run on a StepOnePlus thermocycler (Applied Biosystems). Expression of the gene of interest was normalized to the expression of the ribosomal protein S6 gene (S6), which was measured in parallel as an internal control. Each qPCR reaction product was directly loaded onto nondenaturing 2% agarose gels and visualized under UV transillumination, and specificity of the primers was determined by sequencing the PCR product sequences. The products of the primers used are listed in Table 1. For determination of Sox10 mRNA expression, Taqman expression probe sets were used (Ssox10, Rn00569909_m1; S6, Rn02112097_g1, Applied Biosystems).

Western Blot Analysis

EGC were harvested in RIPA buffer (Upstate-Millipore) containing 2 mM orthovanadate, phosphatase inhibitor cocktail II (Roche, Neuilly sur Seine, France), and a protease inhibitor cocktail (Roche). Tissues were lysed with Precellys 24 tissue homogenizer (Bertin Technologies, Saint-Quentin-en-Yvelines, France), and proteins were extracted using the Nucleospin RNA/Prot kit (Macherey Nagel). Equal amounts of lysate were separated using NuPage Novex Bis-Tris MiniGels (Life Technologies) before electrophoretic transfer with the iBlot Dry Blotting System (Life Technologies). Membranes were blocked for 1 h at 25°C in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris, pH 7.5) with 5% nonfat dry milk or BSA. Membranes were incubated overnight at 4°C with the primary antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham, Les Ulis, France, diluted 1:5,000) and visualized via enhanced chemiluminescent detection (ECL plus, Amersham). The relevant immunoreactive bands were quantified with laser-scanning densitometry and analyzed with ImageJ software. To allow comparison between different films, the density of the bands was expressed as a percentage of the average of controls (untreated). Antibodies included anti-GFAP (1:1,000, Z0334; Dako-Cytomation, Hamburg, Germany), anti-SOX10 (1:500, DAKO), and anti-Ki67 (1:500, DAKO).

Real-Time Quantitative PCR

Total RNA was extracted from rat colonic biopsies or EGC culture using Nucleospin RNA II (Macherey Nagel, Hoerd, Germany). The

Table 1. Real-time PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer, 5′-3′</th>
<th>Reverse Primer, 5′-3′</th>
<th>Accession Number</th>
</tr>
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<tr>
<td>RPS6</td>
<td>CAAGCTTATAGCGCTTCTTGTACTCC</td>
<td>CTCCTGATGCTTCTTGGCG</td>
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<tr>
<td>S100P</td>
<td>GACCTGAGAAGGGGCCATGG</td>
<td>CTATGCTGAGCCTGTTCGAAAGAAGCTC</td>
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<td>GFAP</td>
<td>CCCTGGATGATCCTTTTGC</td>
<td>ATCCTGGCGTGAATGCGCC</td>
<td>NM_017009</td>
</tr>
<tr>
<td>GPR41</td>
<td>CGGCCCTCACAATTGGTGGC</td>
<td>AGGAGATGAAAGGTGGCC</td>
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<td>GPR43</td>
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<td>NM_001005877.1</td>
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<tr>
<td>MCT1</td>
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<td>GATGAGATGAGCAGCAGCA</td>
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<tr>
<td>MCT2</td>
<td>AGATATGAGCCTGATGGA</td>
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<td>MCT4</td>
<td>GAGGGGCACTCCAGTATGGA</td>
<td>AAGTATGAGCCTGATGGA</td>
<td>NM_0308341.1</td>
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Table 2. GFAP expression in colon of rat pups during the postnatal period

<table>
<thead>
<tr>
<th>Age*</th>
<th>Distal Colon Control</th>
<th>Proximal Colon Control</th>
<th>Distal Colon + Butyrate, 2.5 mM</th>
<th>Proximal Colon + Butyrate, 2.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1.0 ± 0.3, n = 11</td>
<td>0.4 ± 0.1, n = 6</td>
<td>2.2 ± 0.6, n = 7</td>
<td>1.9 ± 0.4, n = 7</td>
</tr>
<tr>
<td>P7</td>
<td>2.3 ± 0.4, n = 14</td>
<td>1.7 ± 0.3, n = 8</td>
<td>3.8 ± 0.8, n = 8</td>
<td>3.5 ± 0.3, n = 8</td>
</tr>
<tr>
<td>P14</td>
<td>5.1 ± 0.7, n = 14†</td>
<td>2.7 ± 0.5, n = 8</td>
<td>7.1 ± 1.6, n = 8†</td>
<td>9.8 ± 2.3, n = 8†</td>
</tr>
<tr>
<td>P21</td>
<td>8.8 ± 1.3, n = 14†</td>
<td>7.7 ± 0.9, n = 8†</td>
<td>7.8 ± 1.3, n = 8†</td>
<td>8.4 ± 2.3, n = 8†</td>
</tr>
<tr>
<td>P36</td>
<td>9.1 ± 1.7, n = 14†</td>
<td>6.6 ± 1.3, n = 8†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean GFAP/S6 relative mRNA expression ± SE within the proximal and distal colons of rat pups during the postnatal period, normalized to distal colonic expression at P1. *Postnatal day. †Significant differences in relative expression in distal colon at P1 (Kruskal-Wallis test followed by Dunn’s post hoc test; P < 0.05).

EFFECT OF BUTYRATE ON MYENTERIC GLIAL NETWORK MATURATION

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cytomation), anti-S100β (1:1,000, Z0311; Dakocytomation), anti-Erk1/2 (9102; Cell Signaling), anti-phospho-Erk1/2 (9101; Cell Signaling), anti-acetyl H3k9 (1:1,000, Ab10812; Abcam), and anti-actin (1:5,000, AC-15; Sigma). The relevant immunoreactive bands were quantified with laser-scanning densitometry (ChemiDoc MP System) and analyzed with Image Lab software (Bio-Rad, Courtaboeuf, France). To allow comparison between different scanning images, the density of the bands was expressed as a percentage of the average controls.

Statistical Analysis

Data were expressed as means ± SE. The significance of differences was determined using the Mann-Whitney U-test to compare two groups or the Kruskal-Wallis test to compare more than two groups, followed by Dunn’s multiple-comparison test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Characterization of Myenteric EGC During Postnatal Development of the Rat Colon

To study the postnatal maturation of EGC, the proximal and distal colons of rat pups were dissected at P1, P7, P14, and P21, and expression of the glial markers GFAP and S100β were analyzed by qPCR. Quantitative analyses revealed a time-dependent increase in expression of both markers at P36; levels of GFAP and S100β mRNAs were 9.1- and 5.0-fold higher,

Table 3. S100β expression in colons of rat pups during the postnatal period

<table>
<thead>
<tr>
<th>Age*</th>
<th>Distal Colon Control</th>
<th>Proximal Colon Control</th>
<th>Distal Colon + Butyrate, 2.5 mM</th>
<th>Proximal Colon + Butyrate, 2.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1.0 ± 0.1, n = 11</td>
<td>1.5 ± 0.3, n = 6</td>
<td>3.4 ± 0.7, n = 6</td>
<td>4.3 ± 0.7, n = 7</td>
</tr>
<tr>
<td>P7</td>
<td>4.9 ± 0.6, n = 14†</td>
<td>5.9 ± 1.3, n = 8</td>
<td>6.0 ± 0.3, n = 7†</td>
<td>8.6 ± 1.2, n = 9†</td>
</tr>
<tr>
<td>P14</td>
<td>6.6 ± 1.0, n = 14†</td>
<td>8.3 ± 0.8, n = 7†</td>
<td>6.1 ± 2.3, n = 7</td>
<td>5.5 ± 1.0, n = 8†</td>
</tr>
<tr>
<td>P21</td>
<td>4.1 ± 0.6, n = 14†</td>
<td>9.7 ± 2.2, n = 8†</td>
<td>3.5 ± 0.6, n = 7</td>
<td>6.5 ± 1.3, n = 9†</td>
</tr>
<tr>
<td>P36</td>
<td>5.0 ± 1.3, n = 14†</td>
<td>7.3 ± 1.8, n = 8</td>
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<td></td>
</tr>
</tbody>
</table>

Values represent mean S100β/S6 relative mRNA expression ± SE within the whole-thickness proximal and distal colon of rat pups during the postnatal period, normalized to distal colonic expression at P1. *Postnatal day. †Significant differences in relative expression in distal colon at P1 (Kruskal-Wallis test followed by Dunn’s post hoc test; $P < 0.05$).

Fig. 1. Postnatal maturation of the enteric glial network in distal colonic myenteric plexus. A–C: real-time quantitative PCR (qPCR) showing expression, relative to P1, of the glial markers GFAP (A), S100β (B), and Sox10 (C) during the postnatal period in full-thickness distal colon biopsies of rat pups. Expression levels are normalized to the ribosomal S6 gene in all experiments. *Significant differences relative to expression at P1. †Significant differences relative to expression at P7 (n = 6–14, Kruskal-Wallis test followed by Dunn’s post hoc test, $P < 0.05$). D–K: whole mount preparations of myenteric plexus of rat pup distal colonic specimens at postnatal day 1 (P1) (D, H), P7 (E, I), P21 (F, J), and P36 (G, K). Expression of the glial markers GFAP (D–G) and S100β (H–K) were assessed. Scale bar = 50 μm.
Table 4. Sox10 expression in the colon of rat pups during the postnatal period

<table>
<thead>
<tr>
<th>Age*</th>
<th>Distal Colon Control</th>
<th>Proximal Colon Control</th>
<th>Distal Colon + Butyrate, 2.5 mM</th>
<th>Proximal Colon + Butyrate, 2.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1.0 ± 0.1, n = 3</td>
<td>0.7 ± 0.3, n = 6</td>
<td>1.4 ± 0.3, n = 7</td>
<td>1.1 ± 0.4, n = 7</td>
</tr>
<tr>
<td>P7</td>
<td>1.2 ± 0.8, n = 7</td>
<td>1.3 ± 0.7, n = 8</td>
<td>1.4 ± 0.4, n = 8</td>
<td>1.1 ± 0.4, n = 8</td>
</tr>
<tr>
<td>P14</td>
<td>0.5 ± 0.2, n = 8</td>
<td>0.7 ± 0.5, n = 8</td>
<td>0.4 ± 0.2, n = 8†</td>
<td>0.6 ± 0.3, n = 7</td>
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<tr>
<td>P36</td>
<td>0.4 ± 0.4, n = 8†</td>
<td>0.4 ± 0.3, n = 8†</td>
<td>0.5 ± 0.6, n = 8†</td>
<td>0.3 ± 0.2, n = 8†</td>
</tr>
</tbody>
</table>

Values represent mean Sox10/S6 relative mRNA expression ± SE within the whole-thickness proximal and distal colon of rat pups during the postnatal period, normalized to distal colonic expression at P1. *Postnatal day. †Significant differences in relative expression in distal colon at P7 (Kruskal-Wallis test followed by Dunn’s post hoc test; P < 0.05).

respectively, than at P1 (Table 2, Table 3, and Fig. 1, A and B). We showed that the mRNA expression level of Sox10, which is expressed by ENS progenitors and EGC, decreased progressively over time, being statistically significantly lower at P21 and P36 than at P7 (Table 4 and Fig. 1C). Because no significant difference was observed between the proximal and distal colons (Tables 2–4), the next analyses focused on the distal colon.

The progressive increase in GFAP and S100β expressions during the postnatal period was confirmed at the protein levels by performing immunohistochemical analysis on the myenteric plexus of rat distal colon. At P1, immunoreactivity against GFAP was very weak, making it impossible to clearly distinguish EGC cell bodies. At P7, the labeling was stronger with the appearance of positive staining along the glial processes. At later time points, GFAP labeling further increased, and at P36, a clear network of GFAP-positive cells was observed in the myenteric plexus (Fig. 1, D–G). Changes in cell expression were also observed for S100β. At P1, immunoreactivity against the S100β protein was clearly detected in the myenteric plexus of rat distal colon, but labeling was restricted to cell nuclei (Fig. 1, H–K). At later stages such as P21, S100β expression extended to the cytoplasm, and at P36, immunopositive staining was mostly localized in the cell body.

A comparable evolution was discerned in primary EGC prepared from animals at P14 and P36. As was observed in freshly isolated colons from animals aged P14 and P36, S100β mRNA levels were not significantly different in either group of EGC, but levels of GFAP mRNA were much higher (sixfold) in P36 EGC compared with P14 EGC (Fig. 2, A and B) after 60 days in culture. Despite these phenotypic differences, no change in ATP-induced calcium response was observed between EGC at P14 and P36. The amplitude of ATP-induced Ca2+ transients as well as half-maximum or 10% maximum duration were similar in EGC at P14 compared with those at P36 (Fig. 2, C–F), after normalization to their response to ionomycin.

Characterization and Proliferation of Sox10-IR Cells in Postnatal Myenteric Plexus

The number and proliferating status of Sox10-IR cells were analyzed in the myenteric plexus of the rat distal colon. At P1, the density was 2.5 Sox10-IR cells per 1,000 μm², but it progressively decreased to 1.2 Sox10-IR cells per 1,000 μm² at P36 (Fig. 3, A–D, N). Just after birth, only 78% of the Sox10-IR cells were S100β-IR, however, this proportion increased up to 98% at P7 (P = 0.0002, n = 8, data not shown). To further characterize Sox10-IR cells, double-immunohistochemical analyses were performed with antibodies directed against Sox10 and HuC/D, a neuronal marker. No Hu-IR cells were Sox10-IR (Fig. 3, A, D–N), but we observed that at P1, Sox10-IR cells were distributed in close proximity to Hu-IR cells organized as continuous rows (Fig. 3A). At P7, the majority of Sox10-IR cells were located close to neurons, which started to be organized as individual ganglia. Few Sox10-IR cells were dispersed between these ganglia (Fig. 3, B–D). As indicated in Figure 3, the ratio of Sox10-IR cells to Hu-IR cells increased progressively over time from 0.8 to 1.4, becoming statistically significant at P36 (Fig. 3M). To evaluate the proliferative activity of Sox10-IR cells in the myenteric plexus during the first postnatal weeks, Sox10/Ki67 costaining was...
performed (Fig. 3, E–L). At P1, 12% of Sox10-IR cells were Ki67-IR. This percentage was reduced to 2% at P36 (Fig. 3, E–L and O). No Hu-IR cells were Ki67-IR, and no caspase3 immunoreactivity was observed in Sox10-IR cells at any stages analyzed (data not shown). To determine whether proliferating Sox10-IR cells were already committed to the glial phenotype, triple labeling for S100β/H9252, Sox10, and KI67 was performed at P1 and P7 (Fig. 4, A–H). At P1, 40% of Sox10-IR/KI67-IR cells also expressed S100β. This proportion was significantly increased to 82% at P7 (n = 4–5, Mann-Whitney U-test, P < 0.05).

**Effect of Butyrate on Proliferative Activity and Maturation of EGC In Vivo**

To evaluate the effect of SCFA administration on the proliferative activity and maturation of EGC, rat pups at P5 were treated daily with 2.5 mM butyrate enemas, and their myenteric plexus were collected at P7, P21, and P36. A threefold decrease in the percentage of Sox10/Ki67 double-positive cells was observed as early as P7, indicating an effect of the butyrate treatment on the proliferative activity of Sox10-IR cells (Fig. 5, A–E). Daily administration of 2.5 mM butyrate from P5 onward did not modify mRNA levels of GFAP, S100β, or Sox10 at any of the time point studied (Tables 2, 3, and 4). To determine whether acute administration of butyrate modulates expression of these three glial markers, butyrate enemas were administrated to rat pups at P6 at concentrations of 0, 2.5, 10, or 40 mM, and their effects were analyzed 24 h later. Quantitative PCR and Western blot analyses indicated no significant change in expressions of GFAP, S100β, or Sox10 up to 40 mM of butyrate (Fig. 5, F–I, and data not shown).

**EGC Respond to Butyrate**

To investigate the possibility of a direct effect of butyrate on EGC, expression of its receptors and transporters was analyzed...
in the EGC cell line JUG-2. RT-PCR analyses did not detect MTC4 or GPR43, but JUG-2 cells clearly expressed MCT1 and MCT2, and GPR41 and GPR109A (Fig. 6A). ERK phosphorylation is a major signaling pathway for SCFA receptors. We first showed that butyrate (500 μM) induced an increase in phosphorylated ERK/ERK compared with controls, but similar concentrations of propionate or acetate did not (Fig. 6, B and C). We further performed a dose-response experiment by treating JUG-2 cells with 1, 10, or 100 μM of butyrate for 5 min. Analysis of the ratio of phosphorylated ERK to total ERK showed a significant increase of ERK phosphorylation at a dose of 100 μM (Fig. 6D). Butyrate has been shown to control gene expression by inhibiting histone deacetylase (HDAC). To study such a possible mechanism in EGC, histone acetylation levels were analyzed by immunocytochemistry with an antibody directed against acetylated histone-3 (H3) at lysine 9 (ac-H3K9). H3K9 immunoreactivity in EGC was increased by butyrate treatment compared with control (Fig. 6E). In particular, mean fluorescence intensity of the nucleus was significantly increased by 37.5% after 24 h of treatment with butyrate compared with controls. Western blot analysis further showed that ac-H3K9 expression was increased by 1.8-fold after treatment of JUG-2 cells with butyrate compared with controls (Fig. 6, F and G).

Butyrate Inhibits Proliferation of EGC In Vitro

To confirm that the decreased proliferative activity of Sox10-IR cells in vivo was a direct effect of butyrate administration, JUG-2 cells were treated with 500 μM of the SCFA for 7 days. Cell counting revealed a 61% decrease in the number of cells in the treated cultures compared with control cultures (Fig. 7L). A similar reduction was obtained using trichostatin (TSA), an HDAC inhibitor. The decrease in cell number was associated with a lower number of proliferating cells as determined by Ki-67 immunocytochemistry. Indeed, the percentage of Ki67-IR EGC was 67% lower in butyrate-treated cultures than in control cultures (Fig. 7, A–D and K). This effect was specific because other SCFA such as propionate or acetate, did not affect the number of EGC or the percentage of Ki67-IR cells (Fig. 7, E–H, K, L). On the other hand, incorporation of 7-aminoactinomycin D (7-AAD), a cell death marker, indicated no significant variation in the proportion of living cells between butyrate and nontreated EGC (95% vs. 98%, respectively, data not shown). The effect of butyrate on EGC was further investigated by analyzing its effect on cell cycle progression. As presented in Figure 7 (M–P), 48 h of treatment with butyrate led to a greater accumulation of cells in G1 phase, which was accompanied by a decrease in the number of EGC in S phase. The number of EGC in G2 phase remained unchanged (Fig. 7, M, N, Q). The effect of butyrate on EGC proliferation was further tested on primary cultures of EGC. After treatment with 500 μM butyrate, proliferation of EGC at P14 and P36 was significantly decreased by 28.6 and 53.2%, respectively (Fig. 7, R and S). Expression of MCT1 and MCT2, analyzed by qPCR was similar in EGC at P14 and P36 (data not shown).

DISCUSSION

In the present study we characterized the postnatal development of the myenteric colonic glial network in rat pups. Using Sox10 and Ki67 as markers, we showed a progressive reduction in the number of EGC or progenitors that proliferate in the distal colonic myenteric plexus of rat pups. Although Sox10 and S100β expression may vary in EGC, these markers are generally coexpressed in myenteric EGC and represent the most reliable markers for these cell types (7, 32). At P1, 22%
of Sox10-IR cells were negative for the EGC marker S100β, raising the possibility that not only do EGC proliferate around birth, but do ENS progenitors. This was further supported by triple labeling for Sox10, S100β/H9252, and KI67, which demonstrated that 40% of proliferative Sox10-IR cells were committed to the glial phenotype at this stage. This proportion increased to 82% at P7, supporting the concept that both EGC and a smaller proportion of ENS progenitors are still proliferating at this later stage. Proliferation of EGC under basal conditions was recently described in rodents (8, 19). The rate of proliferation that we observed at 5 wk is consistent with the one described in adult mice (19). The ratio of glial cells to neurons, also called the glia index, increased during the postnatal period to reach 1.4 in adult animals, being thus in range with results reported for guinea pigs (1.7), but smaller than results reported in humans (5.9 to 7.0) (17).

Decreased proliferation in the colonic myenteric plexus during postnatal development goes along with increased expression of glial markers. At P1, S100β/H9252 and GFAP were barely detected, but their expression strongly increased during the first postnatal weeks. At P36, the glial markers were strongly expressed in the glial processes, revealing a glial network similar to those described in adult rodents. These results are consistent with data obtained from mice, in which colonic expression of these markers was not observed before P0 (38). They also fit well with a recent elegant study showing that a specific population of EGC within the mucosa similarly develops after birth in a microbiota-dependent manner (20). Postnatal increase in GFAP was also observed in the human small bowel, indicating that EGC postnatal maturation is a conserved feature in mammals (21). The changes in glial marker expression were associated with particular subcellular distribution. Around birth, the S100β/H9252 protein was detected primarily in the nucleus. This feature is of interest because nuclear accumulation of S100β in oligodendrocyte progenitors has been correlated with cell cycle withdrawal and morphological differentiation (13). In addition, S100β was demonstrated to activate and interact with p53, an essential regulator of cell proliferation (6). Taken together, these pieces of information suggest an active role for this calcium-binding protein in the development of the enteric glial network.

The subcellular distribution of GFAP in EGC also changes during postnatal development. Increased GFAP expression parallels EGC morphological changes and, at P36, we observed similar to those described in adult rodents. These results are consistent with data obtained from mice, in which colonic expression of these markers was not observed before P0 (38). They also fit well with a recent elegant study showing that a specific population of EGC within the mucosa similarly develops after birth in a microbiota-dependent manner (20). Postnatal increase in GFAP was also observed in the human small bowel, indicating that EGC postnatal maturation is a conserved feature in mammals (21). The changes in glial marker expression were associated with particular subcellular distribution. Around birth, the S100β protein was detected primarily in the nucleus. This feature is of interest because nuclear accumulation of S100β in oligodendrocyte progenitors has been correlated with cell cycle withdrawal and morphological differentiation (13). In addition, S100β was demonstrated to activate and interact with p53, an essential regulator of cell proliferation (6). Taken together, these pieces of information suggest an active role for this calcium-binding protein in the development of the enteric glial network.
a glial network with strong GFAP staining in the glial processes. A potential implication for GFAP in glial modeling remains to be determined, but we have shown that primary EGC, which have conserved the age-related changes in GFAP expression, present functional differences. Because expression of GFAP and S100β is regulated in a Ca^{2+}-dependent manner, changes in calcium signaling during postnatal development could participate in regulation of EGC marker expression. We could not detect any difference in ATP-induced Ca^{2+} responses between EGC at P14 or P36. However, we cannot exclude that the culture period (i.e., 60 days) prior the study of EGC responses at P14 or P36. Whether similar differences occur in the developing and mature ENS remains to be determined, but we have shown that primary EGC, which have conserved the age-related changes in GFAP and S100β expression, present functional differences.

In the present study we also provide evidence that SCFA, and in particular butyrate, may affect ENS development, in part through their effect on EGC. Luminal butyrate has been shown to strongly increase during the postnatal period in rats, with a concentration of butyrate in ceco-colonic content ranging from 3 μmol/g of dried feces at P18 to 33 μmol/g at P40 (4). These changes are probably associated with changes in microbiota composition and nutrition during the neonatal period. Furthermore, blood concentration of butyrate as high as 3 mM has been reported in portal blood (9), which is above the concentration used in our study. Interestingly, 100 μM butyrate, which activates pERK/ERK in EGC, has also been shown to increase excitability of after-hyperpolarizing (AH type 2) enteric neurons (26). In our study, butyrate was shown to inhibit proliferation of EGC. Cell accumulation in G1 phase entry into S phase, as has been previously shown in glioma cell lines (18). No induction of cell death was observed upon treatment of EGC with 500 μM butyrate. These observations contrast with data showing that treatment of neonatal astrocytes with butyrate induced cell death (3), but the authors of that study used a higher concentration of butyrate (>1 mM), and the cells were cultured without serum.

The precise mechanisms involved in the inhibitory effects of butyrate on EGC remain to be clarified, but the detection of GPR41, GPR109A, MCT1, and MCT2 in EGC raises the possibility of an action through the seven transmembrane G-coupled receptors and/or transporters. Indeed, we showed in EGC that butyrate resulted in a greater expression of both phosphorylated active forms of Erk-1 and Erk-2 and acetylation of the histone H3, which have been involved in receptor-and transporter-mediated effects of butyrate, respectively. Whether increased acetylation results from inhibition of HDAC activity as observed at high butyrate concentration, or
it involved ATP citrate lyase (ACL)/histone acetyltransferase (HAT) as detected at low butyrate concentration (14b) remains to be determined, but treatment with the HDAC inhibitor TSA similarly inhibited EGC proliferation, and antiproliferative effects of butyrate have previously been associated with its HDAC inhibitor activity (14b). Interestingly, similar changes in levels of histone acetylation are involved in butyrate-mediated regulation of choline acetyltransferase (ChAT) expression in the ENS (35). Histone acetylation processes vary according to SCFA concentration and result in induction of ChAT expression and acetylation of H3K9.

In addition to its effect on EGC proliferation, butyrate could influence EGC maturation because an increase in GFAP expression was described in butyrate-treated C6 rat glioma cells by Morita and coauthors (25). However, here we show that chronic treatment of rat pups with 2.5 mM butyrate or acute treatment with doses up to 40 mM has no effect on expression of GFAP or S100B. These observations are in agreement with the results reported by Hirschfeld and Bressler (16), who showed that butyrate has no effect on GFAP expression in the C6 rat glioma cell line. Whether butyrate affects maturation of EGC in the human colon remains to be determined [a decrease in expression of S100B and GFAP has been described in human-derived glioma cell lines following butyrate treatment (16), suggesting that species-specific differences may exist]. Of interest among these findings is the recent study by Karaboudis et al. (20) showing that microbiota had a central role in favoring the development and maturation of mucosal EGC. Whether butyrate is involved in these effects remains to be determined.

Our present study confirms and extends to the myenteric plexus the concept that profound early postnatal changes occur in the enteric glial phenotype and that it can be modulated by environmental factors such as butyrate. In view of the central role played by EGC in gut health and disease, therapies aimed at enhancing the development of EGC during the postnatal period could be of interest in pathologies of the newborn with altered gut functions. However, before such applications are developed, deciphering the functional consequences of postnatal glial maturation represents an important research axis for future studies.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

REFERENCES


