Postnatal development of myenteric neurochemical phenotype and impact on neuromuscular transmission in the rat colon

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The postnatal period is a key period of life and is particularly sensitive to the influence of various environmental factors. This period is characterized by the maturation of various organs and in particular of the gut. Indeed, various gastrointestinal (GI) functions such as intestinal barrier function or motility continue their maturation and development after birth. This is particularly true in rodents such as rats or mice, making them good models for studying GI dysfunctions observed in preterm infants, such as constipation (2).

Among the key regulators of GI functions is the enteric nervous system (ENS). The ENS has been shown to control GI motility and intestinal barrier functions (22). Cholinergic excitatory motor neurons [identified as choline acetyltransferase (ChAT)-immunoreactive (IR)] and often colocalized with substance P and nitrergic inhibitory motor neurons [identified as neuronal nitric oxide synthase (nNOS)-IR] and often colocalized with vasoactive intestinal polypeptide (VIP), pituitary activating cyclic AMP peptide (PACAP), or adenosine triphosphate (ATP) form two functionally distinct populations of major importance in the control of peristaltic activity (7). Although major effort has been placed to study the ENS during development and in adults, data are still scarce concerning the development of the ENS phenotype and its functional impact during the postnatal period, in particular during the period ranging from birth to weaning. The ENS originates from the vagal and sacral neural crest cells and colonizes the digestive tract during the prenatal period. The entire length of the gut is colonized by embryonic day (E) 8.5 in the chick (11, 27), E14 in the mouse (29), and E16.5 in rat (16). This development of the ENS is also associated with a time-dependent differentiation of specific neurochemically identified neuronal populations that has been described mainly in the prenatal period of mice (28). In rats, 5-HT expression appears early during the embryonic life whereas other mediators appear later such as nitric oxide (NO) by E18, VIP during the suckling period, and PACAP-27 during the weaning period (13). A recent study has also shown an increase in the vesicular acetylcholine transporter (VACHT) immunoreactivity in the mouse pup, which was correlated with the development of colonic migrating motor complexes (CMMCs) (19). In guinea pig, a strong NO-dependent inhibitory component was observed in the ileal longitudinal smooth muscle in neonatal tissues (younger than 2 days postnatal), which was significantly reduced in adult tissues, presumably because of the development of a tachykinergic excitatory component (3).

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Interestingly, in the frog, the development of a cholinergic tone was observed later in the development (24). Similarly, in the zebrafish, the development of tetrodotoxin (TTX)-sensitive motility pattern has been described to occur in the early postfertilization period (10). However, a precise early postnatal characterization of the development of the neurochemical coding and its functional impact on neurally mediated contractile response remains largely unknown.

Therefore, the aims of our study were to analyze the development of the cholinergic and nitrergic phenotype of colonic myenteric neurons during the early postnatal days and to determine ex vivo the functional impact on the neurally mediated contractile response in rat colon.
MATERIALS AND METHODS

Animal Model

The Institutional Animal Care and Use Committee of the University of Nantes approved all the animal studies. Pregnant Sprague-Dawley rats were obtained at 13–14 days of gestation (Janvier Laboratories). Rats were accustomed to laboratory conditions for at least 1 wk before delivery and were individually housed in cages on a 12:12-h light-dark cycle with free access to food and water. Mothers and their pups (12–16 pups/litters) were kept in the same conditions during the whole experiments. Day of birth was considered as postnatal day (P) 0. Pups were euthanized at P1, P3, P5, P7, P14, P21, and P36. Pups were killed by decapitation (P0 to P14) or were anesthetized with isoflurane (5 min; Abbot) and killed by cervical dislocation (P21 to P36).

In Vivo and Ex Vivo Measurement of Motility and Contractile Activity

In vivo. Distal colon transit time was measured by a protocol adapted from studies performed on mice, as previously described (12). A 2-mm diameter glass bead (Sigma) was inserted into the distal colon of rats (5 mm from the anus) by use of a glass rod with a fire-polished end. After bead insertion, rat pups were isolated in their cage without access to food and water. Distal colonic transit was determined in a single rat at a time by monitoring the time required for the expulsion of the glass bead (bead latency) from the time of its insertion. After an hour, if the bead was not eliminated, the experiment was stopped to prevent pup isolation stress and cooling.

Ex vivo. Segments of rat proximal colon (1 cm starting from the cecum) were cleaned of their luminal contents with oxygenated Krebs solution containing (in mM) (117 NaCl, 4.7 KCl, 1.2 MgCl2, 1.2
NaH₂PO₄, 25.0 NaHCO₃, 2.5 CaCl₂, and 11.0 glucose). Proximal colon segments were then attached in the longitudinal direction in an organ bath filled with oxygenated Krebs solution and were initially stretched with a preload of 0.2–0.8 g of tension (depending on the age of the rat) for 60 min. Neuromuscular transmission was studied following electrical field stimulation (EFS) of enteric neurons by using the following parameters: train duration 10 s, pulse frequency 20 Hz, pulse duration 300 μs, and pulse amplitude 10 V. This procedure was repeated three times with 10-min washout periods between stimulations. The contractile response of longitudinal muscle was continuously recorded by use of isometric force transducers (Basile no. 7005, Italy), coupled to a PowerMac Performa 7100/80 computer equipped with the MacLab/4s system (ADI). To characterize the nitric and cholinergic components of the EFS-induced contractile response, N-nitro-l-arginine methyl ester (l-NNAME) and atropine were added to the bath at a final concentration of 5 × 10⁻⁵ and 10⁻⁶ M, respectively. The area under the curve (AUC) of the EFS-induced response was measured throughout the duration of the EFS (10 s). At the end of each experiment, a dose-response curve with carbachol (10⁻¹¹ to 10⁻⁴ M) was performed.

Paracellular Permeability of Proximal Colon in Ussing Chambers

Full-thickness segments of proximal colon were mounted in 2-mm-diameter Ussing chambers (Transcellab). Tissues were bathed on each side with 2 ml of DMEM (Invitrogen) containing 0.1% fetal calf serum (AbCys) continuously oxygenated and maintained at 37°C by gas flow (95% O₂-5% CO₂). After 15 min of equilibration, 200 μl of apical medium was replaced by 200 μl of sulfonic acid fluorescein (578 Dalton) (Invitrogen). The fluorescence level of basolateral aliquots of 200 μl was measured every 30 min during 180 min using a fluorometer (Thermo Electron). The slope of the change of fluorescence intensity over time was determined by using a linear regression fit.

Immunofluorescence Staining

Segments of colon (2 cm from the cecum and directly adjacent to the segment used for motility studies) were fixed in 0.1 M phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA) at room temperature for 3 h at 4°C. Whole mount of longitudinal muscle and myenteric plexus (LMMP) were obtained by removing the circular muscle by microdissection. Whole mounts of LMMP were first permeabilized with PBS-0.1% sodium azide-4% horse serum-Triton X-100 from four animals at age P1 to P11 to test the following order: goat anti-ChAT (1:200; Millipore) and anti-goat Cy3 (carboxymethylindocyanine) (1:500; Jackson ImmunonoResearch); rabbit anti-nNOS (1:1,000; Alexis) and anti-rabbit Cy5 (7-aminomethylcoumarin) (1:500; Jackson ImmunonoResearch) and mouse anti-HuCuHuD (1:200; Invitrogen) and anti-mouse FITC (fluorescein isothiocyanate) (1:500; Jackson ImmunonoResearch). Pictures were then acquired with a digital camera (model DP71, Olympus, France) coupled to a fluorescence microscope (Olympus IX 50) and analyzed with the Cell B software (Soft Imaging System, Olympus). To determine the general neurochemical phenotype an average of 500 neurons were analyzed for each animal and condition. The data are expressed as the percent of ChAT-IR or nNOS-IR neurons normalized to the total number Hu-IR neurons. Cell size was measured with Cell B software (Soft Imaging System, Olympus). For neuronal density measurement, we counted 500 neurons per experimental condition and animals with the objective ×40 (3–4 fields of view at age P1 to ~12 fields of view at age P36).

Morphological Analysis

Pellet-free tubular segments of colon were fixed in 4% PFA and embedded in paraffin. Sections were made and were stained with hematoxylin and eosin. Measurements of longitudinal and circular muscle thickness, colon perimeter, as well as crypt height were performed on five distinct fields of view (×10) from four animals at each postnatal day. The colonic perimeter was estimated by calculating the perimeter of an ellipse using the following formula: \[2\pi\sqrt{(a^2+b^2)/2}\] where the minor axis \(a\) and the major axis \(b\) were measured under the microscope by use of a micrometer scale.

Statistical Analysis

The results were expressed as means ± SE. Statistical differences were determined by paired \(t\)-test or one-way or two-way ANOVA, followed by post hoc test, as appropriate. \(P\) values of 0.05 or less were considered statistically significant.

RESULTS

Postnatal Changes of Colonic Morphology

In a first step, we characterized the morphological changes of the colon occurring over the first 5 wk of life. The body weight of rats increased time dependently from 7.8 ± 0.5 g at P1 to 141.8 ± 12.6 g at P36 (Fig. 1A) and the colon length from 2.7 ± 0.1 cm at P1 to 12.3 ± 1.1 cm at P36 (Fig. 1B). The perimeter of the proximal colon increased significantly over time starting the third postnatal week (Fig. 1C). Furthermore, circular and longitudinal muscular thickness increased over time by factors of 6.6 and 3.8, respectively (Fig. 1, E and F). Finally, the height of the colonic mucosa (base of the crypt to surface epithelium) was increased by a factor of 4.4 (Fig. 1D) during the same time period of observation.

Postnatal Development of Ex Vivo Paracellular Colonic Permeability

The flux of sulfonic acid (578 Da) was measured in an Ussing chamber in colonic segments. No change in sulfonic acid flux was measured between P1 and P21 (Fig. 2).

Postnatal Development of In Vivo and Ex Vivo Motility

In vivo measurement of colonic transit revealed that bead expulsion time was longer than 1 h at P14 for all the pups. Bead expulsion time was 438 ± 343 s at P17 and was...
significantly reduced at P21 (371 ± 246 s) (n = 10–15, t-test; P < 0.0001).

Spontaneous basal motility patterns were assessed in longitudinal colonic muscle segments (Fig. 3A). At P1 and P3, no spontaneous contractile activity was detected in any of the tissues evaluated (n = 8 and 11, respectively). However, at P7, 50% of the segments exhibited randomly occurring spontaneous contraction of low amplitude. Between P14 and P21 ~80% of the segments exhibited spontaneous contractions whereas at P36 spontaneous contractions were detected in all tissues. The frequency (Fig. 3B) and amplitude (Fig. 3C) of spontaneous contractions increased significantly at P36 compared with P7.

Postnatal Development of Myenteric Plexus Morphology

Immunohistochemical analysis with antibodies directed against Hu was performed to define the ganglia and neurons organization and density. We first showed a time-dependent development of ganglia organization. At P1, neurons were organized in continuous rows parallel to the circular muscle and only rarely could individual ganglia be clearly identified (Fig. 4A). Starting at P7, sparse individual ganglia could be detected (Fig. 4B). At P21 and P36, the ENS was organized as a network of individual ganglia connected by interganglionic fiber strands (Fig. 4, C and D). Quantitative analysis showed a

Fig. 3. Postnatal development of ex vivo spontaneous basal motility of colonic longitudinal muscle strips. At P1 (n = 8) and P3 (n = 4), no spontaneous contractile activity could be detected. At P5 (n = 12) few segments and at P7 (n = 8) 50% of the segments exhibited randomly occurring spontaneous contraction of low amplitude. At P14 (n = 6), P21 (n = 10), and P36 (n = 8), segments exhibited rhythmic spontaneous contraction (A). Quantitative analysis of amplitude (B) and frequency (C) of the contractions revealed a significant increase in these parameters at P36 compared with P7 (means ± SE; 1-way ANOVA followed by Bonferroni’s multiple-comparison test; $P < 0.05$ compared with P7).
time-dependent increase in neuronal cell surface area starting at P21 (Fig. 4E). In addition, neuronal cell density was significantly reduced as early as P7 compared with P1 (Fig. 4F).

Postnatal Development of Neurochemical Phenotype in Myenteric Neurons

Triple immunohistochemical staining with antibodies against Hu, ChAT and nNOS was performed on colonic samples with different postnatal age (Fig. 5, A–I). Quantitative analysis showed at P1, few neurons identified with Hu were also ChAT-IR (2.3 ± 1%) (Fig. 5J). The proportion of ChAT-IR neurons remained unchanged until P7. However, at P14, the proportion of ChAT-IR neurons was significantly increased (6.5 ± 1.8%) compared with P1. At P21 and P36 this proportion was significantly larger than at P14 (10.9 ± 2.6 and 12.7 ± 3.4%, respectively). Analysis of the nNOS-IR population revealed a time-dependent increase in the proportion of nNOS-IR neurons starting at P5 compared with P1 (Fig. 5K). However, starting at P5 the proportion of nNOS-IR neurons remained unchanged until P36.

Postnatal Development of EFS-Induced Contractile Response in the Colonic Longitudinal Muscle

Colonic longitudinal muscle segments were stimulated by EFS, and EFS-induced AUC was analyzed in absence or in...
presence of L-NAME and atropine (Fig. 6A). The EFS-induced 
AUC increased significantly over time starting at P14 com-
pared with P1 but remained unchanged thereafter (2.3 ± 1.1 
vs. 0.4 ± 0.2 g·s, respectively; n = 6 and 7, respectively) (Fig.
6B). In presence of L-NAME, EFS-induced AUC was signifi-
cantly increased compared with control only at P36 (3.6 ± 1.8 
vs. 2.5 ± 1.7 g·s, n = 8). In presence of atropine, EFS-induced 
AUC was significantly reduced compared with EFS-induced 
AUC induced in presence of L-NAME starting at P14 (0.4 ± 
0.3 vs. 1.7 ± 1.0 g·s, n = 6) until P36 (n = 8).

DISCUSSION

Our study revealed major modifications of gut morphology 
and functions, in particular colonic motility, during the early 
postnatal period ranging from birth to weaning. These changes 
were associated with an increase in the proportion of myenteric 
nurons that showed NOS or ChAT immunoreactivity and 
ganglia organization.

One of the major results of this study is the identification of 
a postnatal increase in the myenteric cholinergic phenotype and 
a concomitant development of a cholinergic neuromuscular 
transmission. This study further reinforces the role of excita-
tory pathways, of cholinergic (and probably also tachykiner-
gic) origin, in the development of motility during the postnatal 
period both in mammalian and nonmammalian species. Indeed, 
in mice, postnatal development of CMMC appeared to parallel 
the development of cholinergic fibers (identified with VACHT) 
in the circular muscle (19). Similarly, in guinea pig neonates,
excitatory tachykinergic neuromuscular transmission has also been shown to occur later in the postnatal period (3). In addition, EFS-evoked excitatory junction potentials could be evoked in the colon of late fetal (E17) mice (26). However, in these studies, the precise time course of the cholinergic phenotype apparition and functional impact on neuromuscular transmission remained largely unknown. In the present study, we identified that major changes in cholinergic phenotype and neuromuscular transmission occur during the second and third weeks of life in rats. This period of life corresponds in rats to the weaning period (i.e., at 17–21 days after birth) (14) and therefore to a profound modification of their alimentation. Whether changes in nutritional habits are cause or consequence of the phenotypical or functional changes observed remains currently unknown but will be discussed later. Interestingly, cholinergic transmission appears also later in the development of the frog larvae and the changes occur concomitantly with the onset of feeding (24).

The absence of L-NAME-sensitive neuromuscular transmission observed in our study, although nNOS-IR neurons were present, could be due to several reasons. First, it has been shown that longitudinal muscle in the guinea pig received a predominant cholinergic innervation in the small intestine (5) and colon (15), although a small proportion of nNOS-IR neurons also innervate colonic longitudinal muscle (15). Alternatively, L-NAME-sensitive EFS-induced relaxation could have been hard to identify, since the basal tone was maintained low at P1–P7, the tissue was fragile, and limited stretch was applied. Consistently, in the guinea pig, NO-dependent neuromuscular transmission was studied after precontraction of the longitudinal muscle with histamine (3). The absence of L-NAME sensitive response in our study is, however, probably not due to the inability of the muscle to respond to NO since the NO donor sodium nitroprusside induced relaxation in acetylcholine (ACh)-precontracted longitudinal muscle (data not shown). Conversely, the absence of atropine-sensitive neuromuscular transmission in the early postnatal days reported in this study is probably not due to an absence of sensitivity of muscle to acetylcholine since acetylcholine induced a contraction as early as P1 (data not shown). Changes in neuromuscular transmission observed in the longitudinal muscle probably also extend to the circular muscle because 1) cholinergic and nitrergic myenteric neurons innervate circular muscle and 2) a time-dependent increase in the density of VACHT-IR terminals

![Fig. 6. Postnatal development of electrical field stimulation (EFS)-induced contractile response in the colon. Colonic longitudinal muscle strips were submitted to EFS and EFS-induced area under the curve (AUC) was analyzed in absence or in presence of N-nitro-l-arginine methyl ester (l-NAME) and atropine (A). Quantitative analysis showed that the EFS-induced AUC significantly increased starting at P14 compared with P1 in control condition (B, open bars), l-NAME significantly increased EFS-induced AUC only at P36 compared with control (B, solid bars). In contrast, atropine inhibited EFS-induced AUC as early as on P14 compared with the condition in presence of l-NAME (B, shaded bars) (means ± SE; n = 6–10; 2-way ANOVA on repeated measures followed by Bonferroni t-test; *P < 0.05 compared with previous AUC in control condition measures, #P < 0.05 compared with control condition, **P < 0.05 compared with l-NAME condition).](http://ajpgi.physiology.org/)}
was observed in the circular muscle, increasing significantly at the time when spontaneous CMMCs started to occur (19).

Another major finding of the study was the differential time-dependent development of the proportion of nNOS- and ChAT-IR neurons. Although the proportion of nNOS-IR neurons increased within the first postnatal week, it remained constant thereafter. In contrast, the proportion of ChAT-IR neurons did not change during the first postnatal week but started to increase by P14 and thereafter. A similar development of these two populations has also been reported in other species such as mice, in which nNOS-IR neurons appear by E11.5 and ChAT-IR around E18.5 (9), although ACh could be detected between E10 and E12 (20). In contrast, Vannucci and Faussone-Pellegrini (25) showed that the proportion of ChAT-IR cells did not change from P5 to 3 mo postnatal, suggesting that maturation occurred during the period from E18 to P5. This discrepancy could be due to the technique used to evaluate the proportion of ChAT neurons, i.e., sections compared with whole mounts in our study and also to the low number of animals studied (i.e., 2 per group). Interestingly, a similar delay in the expression of ChAT and nNOS has also been reported in zebrafish, in which nNOS expression is present as early as 4 days postfertilization (dpf) and ChAT expression is still absent by 13 dpf, although ChAT is expressed throughout the adult zebrafish intestine (17). The mechanisms responsible for this time-dependent neurochemical plasticity remain currently unknown but could associate both environmental and/or genetic factors. Although establishment of neurochemical coding has been shown to be under the control of various transcription factors such as Phox2b, Sox10, Mash1, Pax3, Hand2, and Hlx (9), the impact of environmental factors on their expression remains largely unknown. Among the putative environmental factors involved during the postnatal period are nutritional factors (related or not to the establishment of the flora). Indeed, during the postnatal period, nutritional changes can induce neurological changes in enteric neurons. In particular, Gomes et al. (8) showed that protein deprivation throughout pregnancy and for 42 days postnatal decreased acetylcholine esterase staining and ChAT-IR in the small intestine compared with normally fed rat pup. Other nutritional factors such as butyrate, which is a short-chain fatty acid produced by bacterial fermentation and whose concentration increases during the early postnatal period (1), has been shown to directly increase the proportion of ChAT-IR but not nNOS-IR neurons in adult (23). Interestingly, neuronal activity that can be modulated by various environmental factors (mechanical stimuli, luminal factors) could also be involved in the postnatal neuroplastic changes observed, in particular as increasing neuronal activity in the ENS-upregulated VIP and tyrosine hydroxylase expression (6), and in E11.5 and E12.5 hindgut explants TTX reduced the number of nNOS neurons (9).

Besides changes in neurochemical phenotype, other changes have been highlighted in this study. In particular, we observed, as others (21), a time-dependent reduction of myenteric neurons density and an increase in neuronal area. These changes occurred in parallel to changes in gut morphology and in particular in colon perimeter, suggesting mechanical factor such as distension involved in the previous changes. Consistent, Brehmer et al. (4) have reported an increase in neuronal cell body in gut hypertrophic segments. Surprisingly, we did not measure any ex vivo changes in sulfonic acid flux across the colonic mucosa in the Ussing chamber. It is tempting to speculate that this net absence of change could be due to a concomitant increase in intestinal epithelial barrier surface (observed in our study) and a reduction in paracellular permeability per surface of intestinal epithelial barrier due to a postnatal maturation of tight junction (18).

In conclusion, our study highlights the profound early postnatal changes in neurochemical phenotype and neuromuscular functions in the colon. This study further sets the basis for targeting cholinergic neurons for the treatment of dysmotility syndrome and in particular constipation in the newborn. Indeed, impaired intestinal or colonic motility is a major problem encountered in preterm newborn or in children with obstructive syndromes such as atresia or laparoschisis. Therefore, therapies aimed at enhancing the “maturation” of the cholinergic phenotype by nutritional approaches such as butyrate could be of great interest in these pathologies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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