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Ciblage nutritionnel du système nerveux entérique dans les pathologies motrices digestives du prématuré

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Abréviations

BEI : barrière épithélio-intestinale
CCN : cellule de la crête neurale
CGE : cellule gliale entérique
CGRP : calcitonin gene-related peptide
ChAT: choline acetyl transferase
CIC : cellules interstitielles de Cajal
CMM : complexes moteurs migrants
Da : Dalton
E: embryonic day
EFS : electrical field stimulation
EPAN : neurones afférents extrinsèques primaires
GDNF : glial cell line-derived neurotrophic factor
GFAP: glial fibrillary acidic protein
GFP: green fluorescent protein
IPAN : neurones afférents intrinsèques primaires
L-NAME : N(G)-nitro-L-Arginine-Méthyl Ester
LPS : lipopolysaccharides
NOS: NO synthase
PND: postnatal day
PPR: pattern- recognition receptor
RET: proto-oncogene tyrosine protein kinase receptor Ret
SNC : système nerveux central
SNE : système nerveux entérique
Sox: SRY related HMG-box
S100β : S100 calcium-binding protein beta
TJ : tight junctions
TLR : toll like receptor
TTX : tétrodotoxine
UFC : unité formant des colonies
VIP: vaso-intestinal peptide
ZO : zonula occludens

Introduction générale

La pratique moderne de la médecine se base sur des preuves scientifiques en opposition avec la médecine empirique, historique. Les décisions médicales tiennent compte désormais des recommandations et des conférences de consensus qui sont le fruit de l'analyse bibliographique. La nécessité de publier des études de niveau de preuve satisfaisant implique les médecins dans des programmes scientifiques élaborés.

Au delà de la validation de pratiques cliniques, l'innovation médicale est un domaine essentiel où les cliniciens doivent collaborer avec des chercheurs, en particulier dans le cadre de programme de recherche translationnelle. Afin de vivre cette interaction et de favoriser le dialogue entre la paillasse du laboratoire et le lit du malade, quoi de plus naturel que de soutenir un doctorat de sciences médicales au cours de ma formation clinique ?

La chirurgie pédiatrique à orientation viscérale est une spécialité dont les champs d'applications sont larges : pathologies pulmonaires, urologiques et digestives, en particulier les anomalies de développement, du prématuré de 500 grammes au jeune adulte de 90 kg. Lors d'un stage d'internat en réanimation néonatale, stage hors-filière, j'ai été frappé par les troubles digestifs du prématuré. Ils se manifestent par des difficultés d'alimentation, des troubles du transit et peuvent aboutir à des pathologies gravissimes comme l'entérocolite ulcéro-nécrosante dont la mortalité avoisine les 25%. Bien que de nombreux travaux scientifiques aient été publiés sur la compréhension des mécanismes impliqués, la prévention ou le traitement des troubles digestifs restent encore une préoccupation quotidienne.

Un axe de recherche innovant dans la compréhension des troubles digestifs du prématuré concerne la place du système nerveux entérique (SNE). En effet, le tube digestif possède son propre système nerveux, réseau de neurones et de cellules gliales qui permet de coordonner et de réguler les différentes fonctions digestives. Chez le prématuré, l'immaturité globale touche également le tube digestif et probablement son chef d'orchestre, le SNE qui pourrait ainsi être une cible thérapeutique d'intérêt

Cette thématique impliquant physiologie, physiopathologie et démarche clinique semble donc particulièrement adaptée à un projet de recherche translationelle.

Objectifs du travail

Cette collaboration entre chercheurs et médecins est centrée sur l'étude du système nerveux entérique lors de la mise en place des fonctions digestives en période néonatale. L'objectif principal était, après avoir déterminé des marqueurs neuro-entériques de maturation digestive, de moduler le phénotype neuro-glial et/ou les fonctions digestives par des facteurs exogènes d'origine nutritionnel.

Organisation du travail scientifique

Ce travail de thèse s'organise en trois parties. Dans un premier temps, une étude bibliographique introduira les concepts étudiés lors des travaux de recherche : développement du tube digestif et des fonctions digestives, maturation du système nerveux entérique, place des facteurs exogènes tels que le microbiote intestinal et ses métabolites dans la maturation post-natale. Ensuite, trois publications scientifiques seront exposées. Enfin, une discussion générale suivie de perspectives permettra de conclure cette thèse.

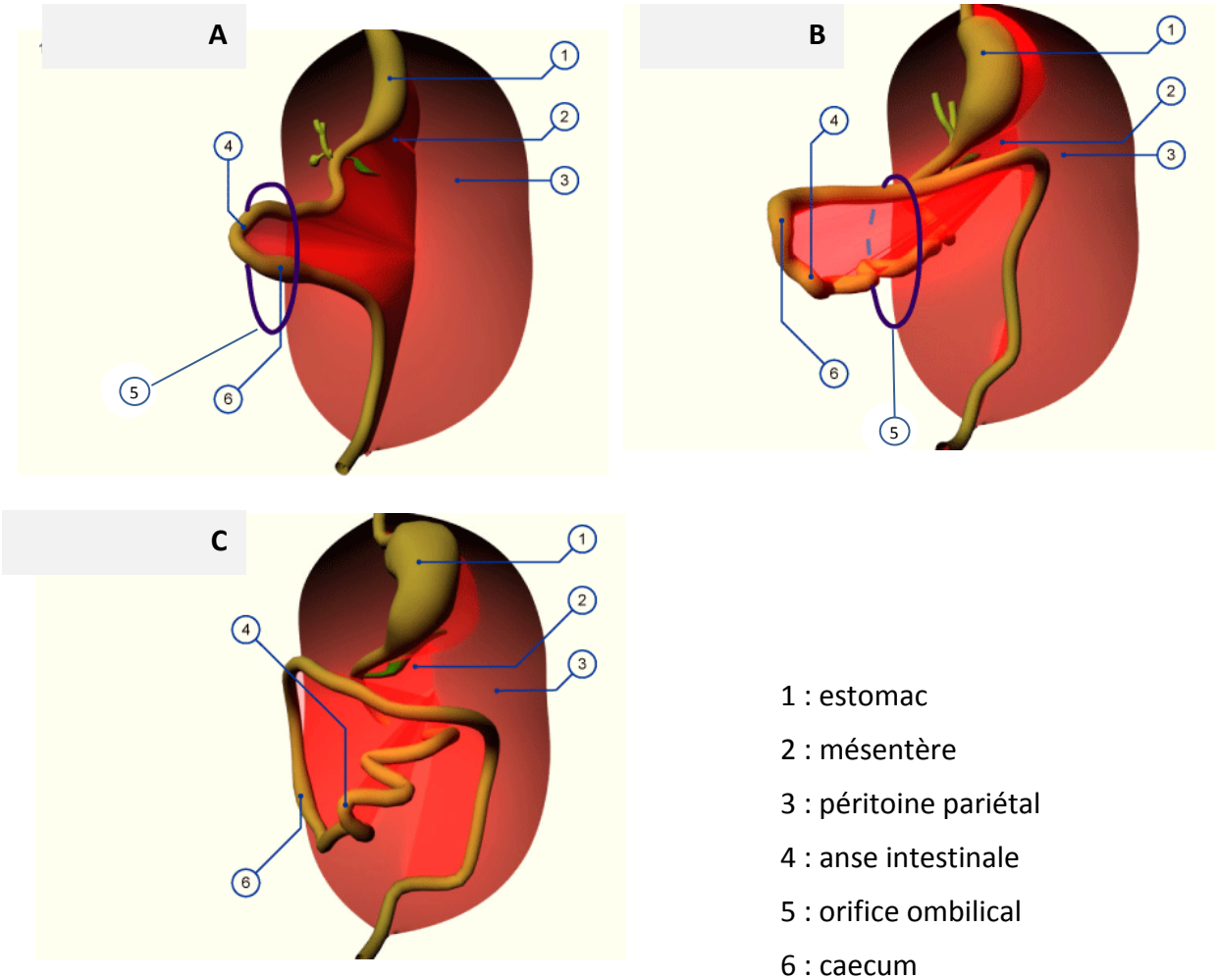
Etude bibliographique

A- Origine et maturation du tube digestif et du système nerveux entérique

1- Développement des structures anatomiques digestives

a. Développement du tractus digestif

L'ébauche primitive du tube digestif est un phénomène précoce du développement. L'embryon, initialement un disque tridermique va présenter deux plicatures : une plicature céphalo-caudale et une plicature latérale. De ces deux plicatures résulte la formation de trois tubes concentriques dont le plus central est l'endoderme, le tube digestif primitif. Il est constitué de trois segments distingués par leur vascularisation : le segment antérieur (foregut) vascularisé par le tronc cœliaque (et deviendra œsophage, estomac et duodénum dans sa partie supra-vaterienne), l'intestin moyen (midgut) vascularisé par l'artère mésentérique supérieure (jéjunum, iléon, côlon droit et moitié droite du côlon transverse) et l'intestin distal (hindgut) vascularisé par l'artère mésentérique inférieure (côlon transverse, côlon gauche et rectum). L'anse intestinale primitive se développe initialement en dehors de l'embryon dans la hernie ombilicale physiologique puis par des phénomènes de croissance différentielle, de réintégration et de rotations, le tractus digestif prend une morphologie définitive à la 12ème semaine de développement (Figure 1).



1 : estomac

2 : mésentère

3 : péritoine pariétal

4 : anse intestinale

5 : orifice ombilical

6 : caecum

Figure 1 : Ebauche et rotations de l'anse intestinale primitive.
L'anse intestinale primitive herniée dans l'orifice ombilicale présente une première rotation de 90° dans le sens anti-horaire vers la fin de la 6ème semaine de développement (A). A la fin de la 10ème semaine, après l'individualisation du jéjunum, de l'iléon et du côlon (B), le tube digestif amorce une réintégration dans la cavité abdominale tout en présentant une rotation de 180° dans le sens anti-horaire dans un plan frontal (C) (http://www.embryology.ch/anglais/sdigestive/mitteldarm01.html).

La paroi du tube intestinal primitif est constituée initialement d'une simple couche de cellules épithéliales endodermiques qui vont proliférer induisant une obstruction complète de la lumière digestive à la 6ème semaine de développement. Des protrusions mésenchymateuses vont se former pour aboutir à la formation des villosités intestinales. Le tractus se perméabilise à nouveau au cours de la 8ème semaine de développement. Le revêtement muqueux prend sa forme définitive à partir de la 9ème semaine. Les autres constituants de la paroi digestive (sous-muqueuse, la couche musculaire et la séreuse) proviennent du mésoderme de voisinage de l'anse intestinale primitive *(embryologie humaine, Larsen ed. De Boek)*.

b. Morphologie définitive du tube digestif

La paroi du tube digestif se compose de 4 tuniques concentriques, respectivement de dedans en dehors: la muqueuse, la couche sous-muqueuse, la musculeuse puis la séreuse. Cette disposition est respectée tout au long du tube digestif (Figure 2).

Chaque segment du tube digestif est voué à une fonction particulière dans la digestion et présente des spécificités anatomiques, morphologiques et cellulaires distinctes. Par exemple, les nutriments, l'eau et les électrolytes sont absorbés au niveau de l'intestin grêle, où la muqueuse montre une surface importante par la présence de replis transversaux ou valvules conniventes, de villosités intestinales et la présence des bordures en brosse des entérocytes. La muqueuse colique comporte des invaginations cryptiques afin de réabsorber l'eau et les sels solubles transformant ainsi les résidus du bol alimentaire en fèces.

L'épithélium intestinal comporte quatre types cellulaires prédominants : les cellules absorbantes ou entérocytes, les cellules mucosécrétantes ou caliciformes à mucus, les cellules entéro-endocrines et les cellules de Paneth. Cet épithélium repose sur la lamina propria ou chorion constitué de tissu conjonctif lâche qui lui aussi repose sur la muscularis mucosa (couche fine de cellules musculaires lisses organisées longitudinalement et circulairement).

La sous-muqueuse est constituée de tissu collagène lâche. Elle soutient la muqueuse et contient des capillaires sanguins, des lymphatiques et des filets nerveux.

La musculeuse est constituée de cellules musculaires lisses, disposées en une couche circulaire interne, et une couche longitudinale externe. Ces deux couches perpendiculaires entre elles, sont responsables de la motricité digestive.

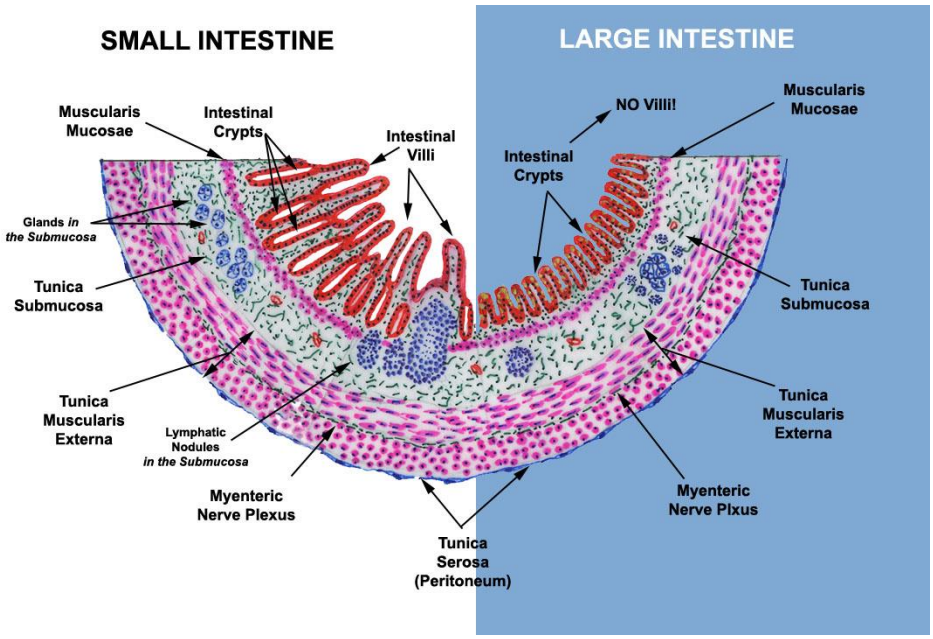


Figure 2 : Coupe schématique comparant la paroi iléale et colique.
La paroi du tube digestif se compose de la séreuse (externe), de la paroi musculaire, de la sous-muqueuse et enfin de la muqueuse. Bien que des similitudes structurelles existent entre les différents segments digestifs, chaque organe possède quelques spécificités. La muqueuse de l'intestin grêle présente des villosités alors que celle du côlon, des cryptes (site internet: Learn).

2- Le système nerveux entérique

a. Organisation du système nerveux entérique

Le système nerveux entérique (SNE) mature comporte chez l'homme plus de 100 millions de neurones (Goyal and Hirano 1996) et 400 millions de cellules gliales (Rühl, Nasser and Sharkey 2004). Il s'étend sur l'ensemble du tractus digestif, de l'œsophage au rectum. L'agencement de ces cellules est complexe, et diffère en fonction des espèces et de l'organe. Chez l'homme il existe principalement deux réseaux parallèles appelés plexus (Figure 3) : le plexus sous-muqueux de Meissner (présent dans l'intestin grêle et le côlon) et le plexus myentérique. Des plexus sous-muqueux supplémentaires ont également été décrits : le plexus de Schabadash plus profond et un plexus intermédiaire (Timmermans *et al.* 1997).

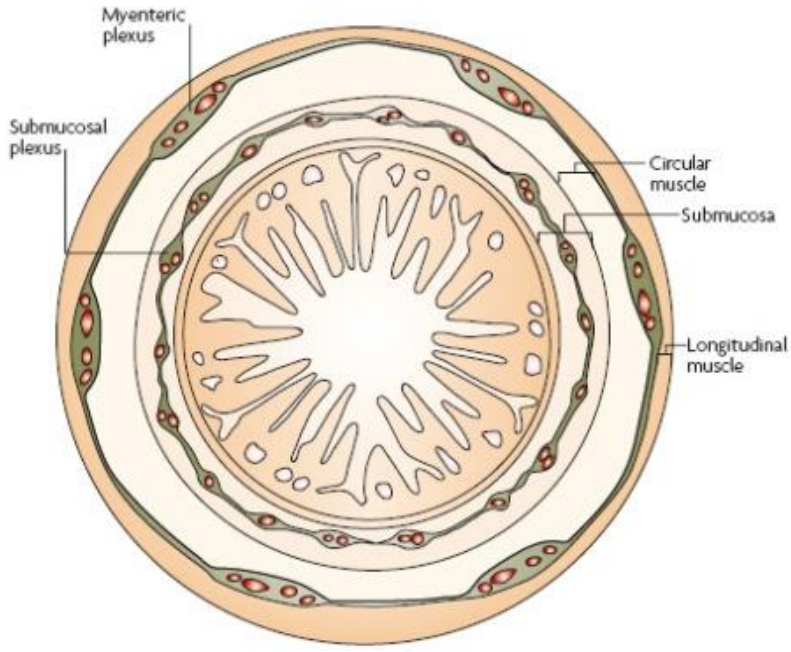


Figure 3: Représentation schématique des plexus sous-muqueux et myentériques sur coupe transversale d'intestin.
Le SNE est constitué de ganglions organisés en deux plexus principaux : le plexus myentérique situé entre les couches musculaires circulaires et longitudinales et le plexus sous-muqueux (Heanue and Pachnis 2007a).

i. Les neurones entériques

Différentes classifications sont utilisées pour décrire les neurones entériques en fonction de leurs morphologies, leurs propriétés électrophysiologiques, leurs fonctions, ou leurs contenus en neuromédiateurs. La classification descriptive de Dogiel (Dogiel 1899) complétée par Furness et Timmermans (Furness, Bornstein and Trussell 1988; Timmermans *et al.* 1997) propose sept morphotypes différents classés de I à VII (Figure 4).

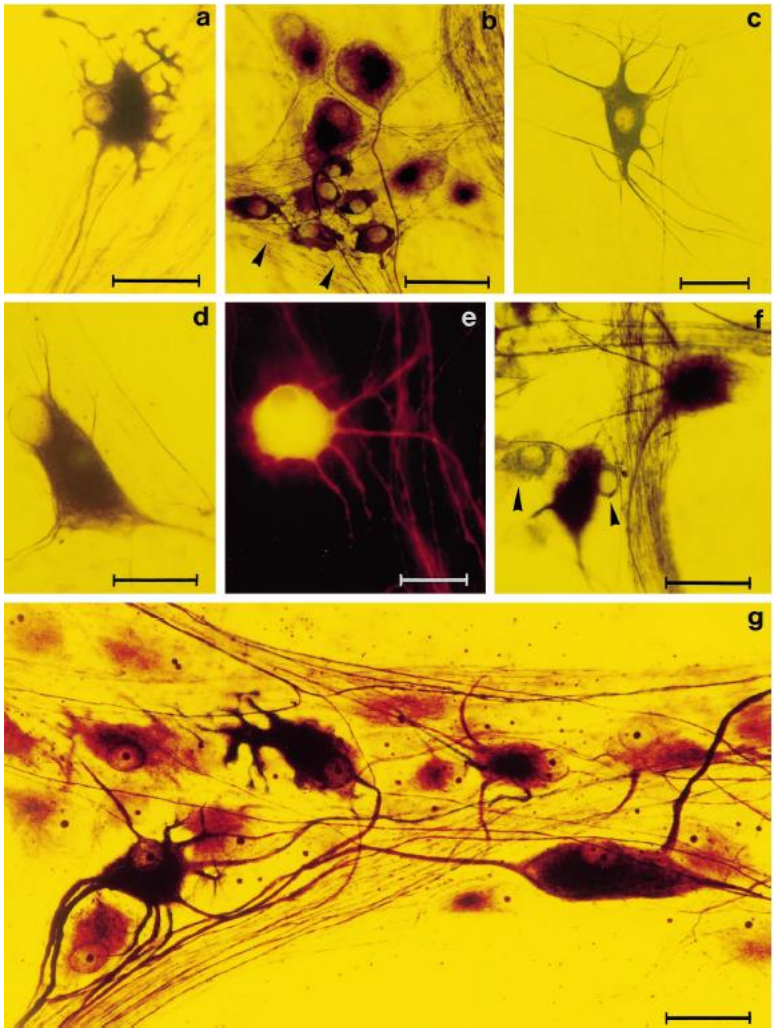


Figure 4: Morphotypes des neurones entériques selon Dogiel.
Les neurones entériques d'intestin de porc en coloration argentique (échelle 50µm). Sept morphotypes différents sont distingués en fonction de la forme du corps cellulaire, du noyau et des prolongements cellulaires (Brehmer, Schrodl and Neuhuber 1999).

L'approche électrophysiologique des neurones entériques distingue deux types de neurones : les neurones de type S ou "Synaptic" et les neurones de types AH ou "After Hyperpolarisation" (Hirst, Holman and Spence 1974).

Le phénotype neuro-chimique du SNE est caractérisé par le contenu des neurones en neuromédiateurs. Les neuromédiateurs du SNE permettent la transmission de l'information vers d'autres neurones mais également vers les cellules effectrices (muscle). Ces neuromédiateurs peuvent être schématiquement classés en fonction de leur action excitatrice ou inhibitrice sur leurs cellules cibles (Tableau 1 et 2). Certains neurones peuvent contenir plusieurs neuromédiateurs (Schemann and Neunlist 2004). Cette approche permet une étude fonctionnelle par l'utilisation d'agonistes ou d'antagonistes pharmacologiques.

Stimulation	Inhibition
Acétylcholine Adénosine Sérotonine Histamine Neurokinine A Substance P Cholecystokinine GRP (gastrin releasing polypeptide) Motiline Bombésine Opioïdes TRH (thyrotropin releasing hormone) PGE2 (prostaglandine E2)	NO VIP (vaso-intestinal peptide) Somatostatine GABA (gamma butyric acid) CGRP (calcitonine gene regulated peptide) NPY (neuropeptide Y) Galanine Glucagon Neurotensine PACAP (pituitary adenylate cyclase activating polypeptide) PHI (peptide histidine isoleucine) PYY (peptide YY) Sérotonine Dopamine

Tableau 1: Principaux neuro-transmetteurs et substances neuro-humorales.
Selon Hansen (Hansen 2003).

Type of neuron	Primary transmitter	Secondary transmitters, modulators	Other neurochemical markers	Study
Enteric excitatory muscle motor neuron	ACh	Tachykinin, enkephalin (presynaptic inhibition)	Calretinin, γ-aminobutyric acid	Brookes *et al.* (1991);[76] Holzer & Holzer Petsche (1997);[77] Grider (2003)[78]
Enteric inhibitory muscle motor neuron	Nitric oxide	VIP, ATP or ATP-like compound, carbon monoxide	PACAP, opioids	Fahrenkrug *et al.* (1978);[79] Costa *et al.* (1992);[80] Sanders & Ward (1992);[81] Xue *et al.* (2000)[82]
Ascending interneuron	ACh	Tachykinin, ATP	Calretinin, enkephalin	Brookes *et al.* (1991)[83]
ChAT, NOS descending interneuron	ATP, ACh	ND	Nitric oxide, VIP	Young *et al.* (1995);[84] Brookes (2001)[85]
ChAT, 5-HT descending interneuron	ACh	5-HT, ATP	ND	Furness & Costa (1982);[86] Monro *et al.* (2002);[87] Gwynne & Bornstein (2007)[88]
ChAT, somatostatin descending interneuron	ACh	ND	Somatostatin	Gwynne & Bornstein (2007);[88] Portbury *et al.* (1995)[89]
Intrinsic sensory neuron	ACh, CGRP, tachykinin	ND	Calbindin, calretinin, IB4 binding	Grider (2003);[78] Gwynne & Bornstein (2007);[88] Li & Furness (1998);[90] Johnson & Bornstein (2004)[91]
Interneurons supplying secretomotor neurons	ACh	ATP, 5-HT	ND	Suprenant (1984);[92] Monro *et al.* (2004)[93]
Noncholinergic secretomotor neuron	VIP	PACAP	NPY (in most species)	Cassuto *et al.* (1981);[94] Banks *et al.* (2005)[95]
Cholinergic secretomotor neuron	ACh	ND	Calretinin	Brookes *et al.* (1991);[83] Keast *et al.* (1985)[96]
Motor neuron to gastrin cells	GRP, ACh	ND	NPY	Holst *et al.* (1987);[97] Weiget *et al.* (1997)[98]
Motor neurons to parietal cells	ACh	Potentially VIP	ND	Nilsson *et al.* (1972);[99] Feldman *et al.* (1979)[100]
Sympathetic neurons, motility inhibiting	Noradrenaline	ND	NPY in some species	Finkleman (1930);[101] Macrae *et al.* (1986)[102]
Sympathetic neurons, secretion inhibiting	Noradrenaline	Somatostatin (in guinea pig)	ND	Costa & Furness (1984)[103]
Sympathetic neurons, vasoconstrictor	Noradrenaline, ATP	Potentially NPY	NPY	Dresel & Wallentin (1966);[104] Furness (1971);[105] Furness *et al.* (1983)[106]
Intestinofugal neurons to sympathetic ganglia	ACh	VIP	Opioid peptides, CCK, GRP	Crowcroft *et al.* (1971);[107] Dalsgaard *et al.* (1983);[108] Love & Szurszewski (1987)[109]

Tableau 2: Classification neurochimique des neurones entériques.
5-HT: 5-hydroxytryptamine, ACh : acétylcholine, ATP : adenosine tri-phosphate, CGRP: calcitonine gene-related peptide, GRP: gastrine releasing peptide, ND: not determined, NPY: neuropeptide Y, PACAP: pituitary adenylyl-cyclase activating peptide, VIP: vaso-intestinal peptide (Furness 2012).

ii. Les cellules gliales entériques

Les cellules gliales entériques (CGE) sont des cellules plus petites que les neurones, présentent de nombreux prolongements cellulaires et ont des formes et tailles variables. Elles sont en contact direct avec les neurones qu'ils soient dans des ganglions ou dans des filets nerveux. Les CGE sont dépourvues de myéline. Plus nombreuses que les neurones, par exemple deux fois plus nombreuses que les neurones dans les ganglions myentériques de l'iléon du cobaye (Gabella 1981), les CGE sont comparées aux astrocytes du système nerveux central par de nombreuses similarités (Figure 5) : elles expriment des marqueurs de la lignée gliale, la glial fibrillary acidic protein (GFAP) (Jessen and Mirsky 1980), la S100 calcium-binding protein beta (S100β) (Ferri *et al.* 1982), la glutamine synthetase (Jessen and Mirsky 1983), et la brain-type fatty acid-binding protein (BFABP). Elles expriment également, moins spécifiquement, le SRY related HMG-box (Sox) 8/9/10 (Hoff *et al.* 2008), tout comme les progéniteurs des cellules dérivées de la crête neurale.

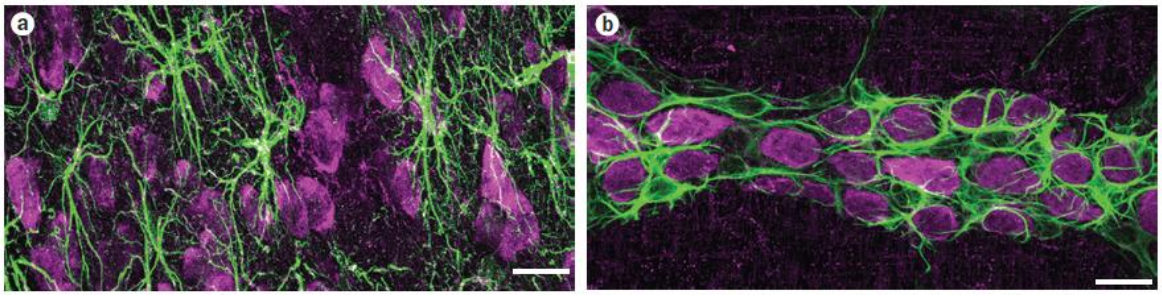


Figure 5 : Astrocytes et cellules gliales entériques.
Les astrocytes du SNC (GFAP immunoréactifs, en vert) s'organisent en étoile autour des neurones (NeuN immunoréactifs, en magenta) sur cette coupe de cortex murin (A) tout comme les cellules gliales entériques du plexus myentérique de côlon murin (neurones Hu immunoréactifs, en vert) (B). Echelle 20µm (Gulbransen and Sharkey 2012).

Les CGE se distribuent dans le tractus digestif et sont distinguées en fonction de leur localisation au sein de la paroi digestive (Gulbransen and Sharkey 2012): muqueuses, sous-muqueuses, musculaires et intra-musculaires (Figure 6). Les CGE muqueuses ont un rôle important dans la maintenance de la barrière épithélio-intestinale (Bush *et al.* 1998; Van Landeghem *et al.* 2009). Les CGE intra-ganglionnaires (sous-muqueuses et musculaires) participent à la gliogenèse et la neurogenèse (Laranjeira *et al.* 2011; Gulbransen and Sharkey 2012; Boesmans *et al.* 2013) tout comme les astrocytes de la zone sub-ventriculaire des ventricules latéraux et les cellules gliales de la rétine (Ooto *et al.* 2004; Stutz *et al.* 2014).

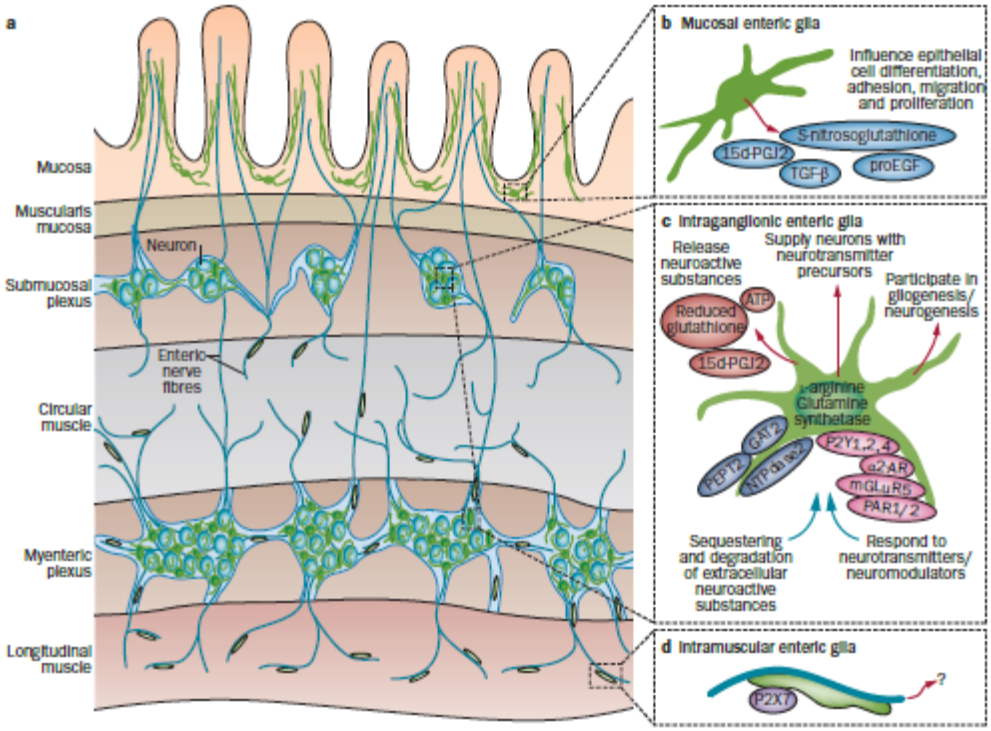


Figure 6 : Classification topographique des cellules gliales entériques.
Représentation schématique des différentes localisations des cellules gliales entériques (A). Sont distinguées les cellules gliales muqueuses (B), intra-ganglionnaires (C), intra-musculaires (D) (Gulbransen and Sharkey 2012).

iii. Connexions et projections du système nerveux entérique

Les prolongements neuronaux se connectent à l'ensemble des constituants du tube digestif : cellules épithéliales, structures vasculaires, cellules endocrines. Par ailleurs, les connexions du SNE se prolongent en dehors du tube digestif par l'intermédiaire des ganglions cœliaques, mésentériques et sympathiques (Figure 7 : Connections et projections du système nerveux entérique.

). L'influx nerveux quitte le tube digestif par des neurones intestinofuges, les EPAN (neurones afférents extrinsèques primaires) vers le système nerveux central (SNC). Les informations en provenance du SNC atteignent le SNE et les effecteurs digestifs par l'intermédiaire des systèmes vagaux, sympathiques et pelviens et des IPAN (neurones afférents intrinsèques primaires) (Furness 2012).

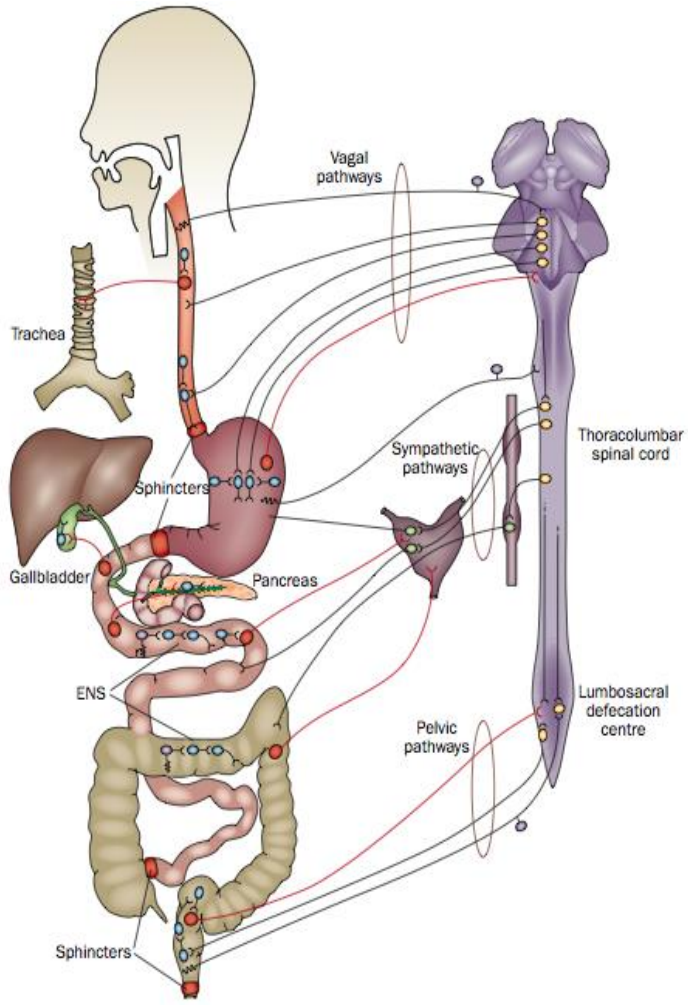


Figure 7 : Connections et projections du système nerveux entérique.
Les neurones entériques (moteurs bleus, sensitifs mauves) permettent le fonctionnement autonome du tube digestif. Les connections extra-digestives se font via les neurones intestinofuges (rouges) vers le système nerveux central (jaune), les ganglions sympathiques, la vésicule biliaire et le pancréas. Les neurones des ganglions para-vertébraux sympathiques (verts) reçoivent des informations du SNC et du SNE. Les informations sensorielles se dirigent d'une part vers le SNE via les IPAN : neurones afférents intrinsèques primaires (mauves), et d'autre part vers le SNC, via les EPAN : neurones afférents extrinsèques primaires (mauves) suivant les circuits afférents spinaux et vagaux. Les informations en provenance du SNC atteignent le SNE et les effecteurs digestifs par l'intermédiaire des systèmes vagal, sympathique et pelvien (Furness 2012).

b. Développement du système nerveux entérique

i. Origine des cellules du SNE : les cellules de la crête neurale

Le système nerveux entérique (SNE) a la particularité d'avoir une origine embryologique différente du tube digestif. La plaque neurale, épaississement médial de l'ectoplasme, se tubulise à partir de la 4ème semaine de développement. Lors de ce phénomène appelé neurulation, un contingent cellulaire se distingue des lèvres latérales de la plaque neurale et constitue les crêtes neurales. Différents contingents sont distingués en fonction de leur localisation : crânial, troncal, vagal, sacré. Ces cellules de la crête neurale (CCN) migrent, colonisent divers organes et se différencient pour participer à la formation du squelette crânien, de l'encéphale, du cœur, des mélanocytes, des ganglions sympathiques et de la surrénale (médullaire) (Figure 8).

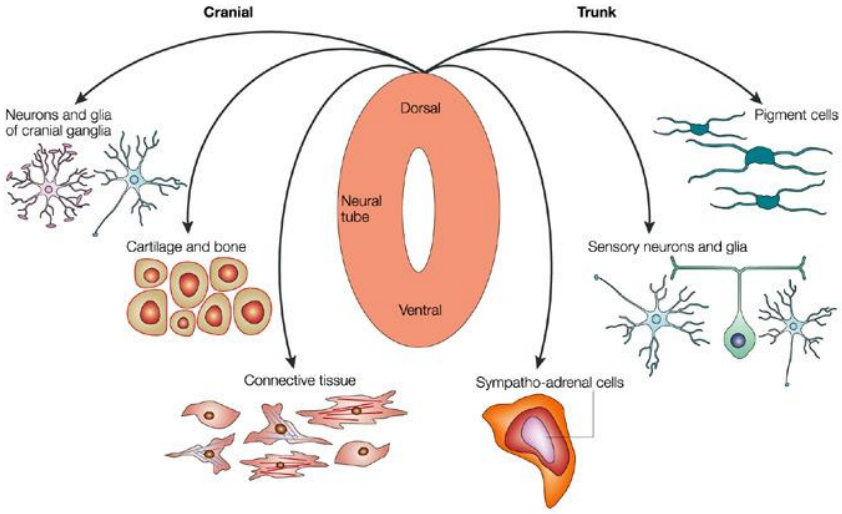


Figure 8 : Devenir des cellules de la crête neurale.
Les cellules de la crête neurale proviennent d'un épaississement du tube neural et sont à l'origine de nombreux types cellulaires comme les neurones, les cellules gliales, la surrénale (médullaire), les mélanocytes (Knecht and Bronner-Fraser 2002).

L'implication des CCN dans la formation du SNE a été étudiée initialement dans les années 1950 où Yntema et Hammond (Yntema and Hammond 1954) ont montré que l'ablation du contingent vagal de la crête neurale aboutissait à une aganglionose totale du tube digestif, c'est-à-dire l'absence de ganglions neuronaux. Dans les années 1970, Le Douarin a utilisé des chimères caille-poulet afin de tracer la migration des CCN (Le Douarin and Teillet 1973). Ces expériences ont montré que l'essentiel des CCN qui colonise le tube digestif provient des somites 1 à 7 (Figure 9).

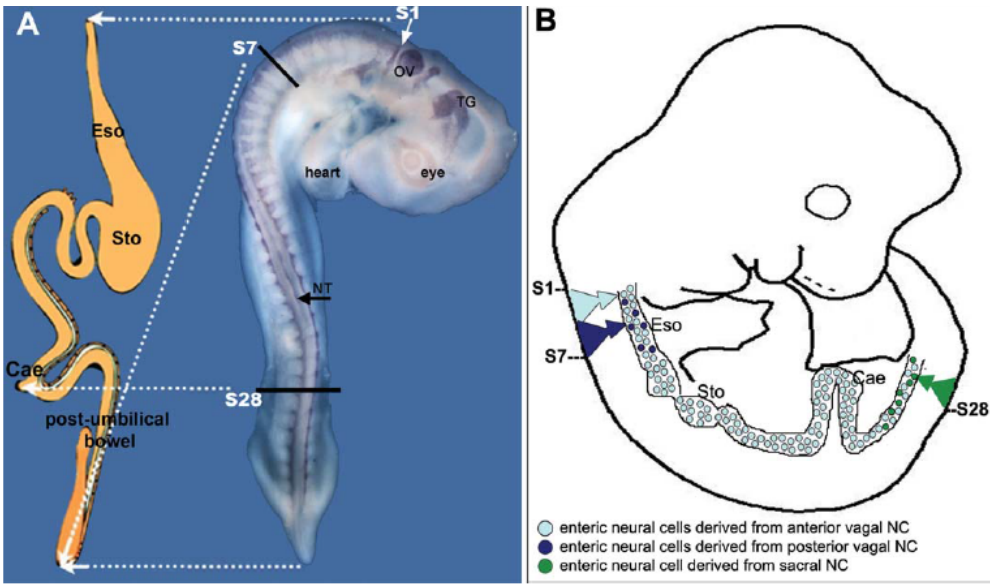


Figure 9 : Migration des cellules de la crête neurale à l'origine du système nerveux entérique.
Une hybridation *in situ* détectant Sox10 sur un embryon de poulet de 3 jours montre la localisation des cellules dérivées de la crête neurale (A). Sur l'embryon de souris schématisé au 14ème jour embryonnaire (B), les cellules dérivées de la crête neurale des somites 1 à 7 colonisent le tube digestif. Le segment sacral de la crête neurale (après le somite 28) contribue à la colonisation du segment post-ombilical du tube digestif (Eso : œsophage, Sto : estomac, Cae: caecum, NT: tube neural, OV: vésicules optiques, TG : ganglion trigéminal) (Coelho-Aguiar *et al.* 2015).

La migration des CCN s'initie par les arcs brachiaux, la partie pharyngée de l'embryon, puis se poursuit dans le tube digestif dans un sens oro-anal (Burns 2005). Ainsi, l'ensemble du tube digestif est colonisé au 8.5^{ème} jour embryonnaire (E8.5) chez le poulet, 14ème jour chez la souris (Kapur, Yost and Palmiter 1992; Burns and Douarin 1998; Young *et al.* 1998a; Wallace and Burns 2005; Olden *et al.* 2008). Chez l'homme, la migration des CCN atteint l'intestin moyen à la 5ème semaine de développement et l'intégralité du tube digestif à la 7ème semaine de développement.

La migration rostro-caudale a été inversée lors d'expériences de greffes de CCN vagales en lieu et place des CCN sacrées (Burns, Delalande and Le Douarin 2002). Dans l'expérience inverse, la greffe de CCN sacrées à la place de CCN vagales a induit une colonisation plus faible et incomplète du tube digestif. Pour expliquer cette différence, les auteurs de cette étude ont montré que le niveau d'expression de RET était plus élevé dans les CCN vagales que dans les CCN sacrées (Schuchardt *et al.* 1994) (voir ci-après).

Le contingent sacré de la crête neurale pourrait également participer à la formation du SNE, notamment chez la souris et le poulet (Le Douarin and Teillet 1973). Ces cellules ne coloniseraient pas l'intestin directement mais via des ganglions nerveux des plexus sacrés (Kapur 2000; Anderson, Stewart and Young 2006; Nagy *et al.* 2007). La proportion de neurones issue de cette voie n'a pas été clairement identifiée chez l'homme.

ii. Mécanismes impliqués dans la migration des CCN

Initialement étudiés dans la maladie de Hirschsprung, les mécanismes impliqués dans la mise en place du SNE concernent la migration et la prolifération des CCN dans et le long du tube digestif en développement, la différenciation neuronale et gliale, la formation de ganglions. Ces phénomènes sont le plus souvent concomitants et interdépendants.

Prolifération et migration

Le premier mécanisme conditionnant une colonisation normale du tube digestif par les CCN serait la taille du pool de progéniteurs de CCN (Barlow *et al.* 2008). Ainsi, l'ablation de certaines régions des CCN vagales a induit une aganglionose partielle chez le poulet, résolutive par greffe de CCN de cailles (Barlow *et al.* 2008).

Par ailleurs, un régulateur majeur des phénomènes de prolifération et de migration est le système GDNF/RET : glial cell line-derived neurotrophic factor/proto-oncogene tyrosine protein kinase receptor Ret (Heanue and Pachnis 2007b)(Figures 10 et 12). RET a été détecté chez la souris précocement dans le développement du tube digestif, dès E9-E11.5 (Pachnis, Mankoo and Costantini 1993; Natarajan *et al.* 2002). RET est un récepteur à tyrosine kinase exprimé par les CCN et ses facteurs de transcription sont Sox10, Phox2B (Southard-Smith, Kos and Pavan 1998; Pattyn *et al.* 1999). Sa mutation, ou sa réduction d'expression, seraient une cause d'aganglionose du tube digestif (maladie de Hirschsprung). Il a été montré que RET possédait également des régulateurs intracellulaires négatifs comme Spry-2 et KIF26A (kinesine like protein) limitant une production neuronale excessive. La délétion homozygote chez la souris de Spry-2 ou de KIF26A a permis d'entraîner une hyperplasie nerveuse entérique responsable d'achalasie œsophagienne ou de pseudo-obstruction intestinale chronique (Taketomi *et al.* 2005; Zhou *et al.* 2009). De manière similaire, la délétion de Pten a induit une hyperganglionose chez la souris responsable de pseudo-obstruction intestinale chronique (Puig *et al.* 2009).

Le ligand préférentiel de RET est le GDNF qui est produit par le mésenchyme du tube digestif. La vitesse de prolifération des CCN est plus importante au niveau du front de

migration qu'au niveau de la queue, conséquence du gradient d'expression de GDNF par le mésenchyme. Wang *et al*. ont montré qu'un excès de GDNF (injection systémique) induisait une augmentation importante de la densité des neurones sous-muqueux chez la souris, alors que l'hyper-expression du GDNF par les cellules gliales (souris GFAP-GDNF$^{+/+}$) augmentait essentiellement la densité des neurones myentériques (Wang *et al.* 2010). La densité neuronale dans le SNE a été réduite à l'extrême chez les souris GDNF$^{-/-}$ et de 30 à 50% chez les souris GDNF$^{+/-}$ (Gianino *et al.* 2003). Un autre mécanisme régulateur est l'inhibition de contact permettant de réguler la densité cellulaire (McClatchey and Yap 2012). De nombreux autres facteurs sont impliqués dans le développement du SNE (Tableau 3 et 4, Figure 10).

Secreted ligand	Role in ENCCs	Phenotype of mouse ENS after perturbation	Evidence for role in Hirschsprung disease
GDNF (mesenchymal)	Promotes survival, proliferation, differentiation and migration[146,147]	*Gdnf*$^{+/-}$: reduction of enteric neurons[2] *Gdnf*$^{-/-}$: aganglionosis distal to the stomach[146]	Yes[148]
Endothelin-3 (mesenchymal)	Promotes proliferation and migration; inhibits differentiation[28,149]	*Edn3*$^{+/-}$: normal enteric neuron density[150] *Edn3*$^{-/-}$: no neurons in the distal colon[151]	Yes[9,152]
ECE1 (mesenchymal)	Processes endothelins to active peptides[153]	*ECE1*$^{-/-}$: lack of neurons in the distal colon[153]	Yes[154]
Neurotrophin-3 (mesenchymal)	Promotes survival and differentiation of developing neurons[87]	*NT3*$^{-/-}$: region-specific decrease in enteric neuron number[87]	ND
Sonic hedgehog (epithelial)	Promotes proliferation and concentric patterning[22,25,155]	*Shh*$^{-/-}$: increased number of neurons in mucosa[156]	ND
Indian hedgehog (epithelial)	Promotes survival of a subpopulation of ENCCs[156]	*Ihh*$^{-/-}$: absence of neurons from parts of small intestine and colon[156]	ND
BMP2 and BMP4 (mesenchymal)	Promotes migration, differentiation, ganglion formation and concentric patterning[93,157]	Antagonism of BMP4: increase or decrease of neuronal subpopulations; overexpression of BMP4: increase in glia–neuron ratio[158,159]	ND
Netrin (epithelial)	Required for secondary migration of ENCCs from the myenteric to submucosal region[160]	Netrin is a ligand for DCC receptors, which are expressed by ENCCs. *DCC*$^{-/-}$ mice lack submucosal ganglia (Table 2)	ND
Semaphorin 3A (mesenchymal)	Delays entry of axons and sacral ENCCs into the hindgut[37,161]	*Sema3A*$^{-/-}$: premature entry of sacral ENCCs[37]	Yes[162]
Neurturin (mesenchymal)	Promotes neurite outgrowth[86]	*Nrtn*$^{-/-}$: defects in submucosal neuron number and excitatory nerve fibre density[2,163]	Yes[164]
GGF2	Promotes glial development[159]	ND	ND

Tableau 3 : Facteurs mésenchymateux et épithéliaux impliqués dans le développement du système nerveux entérique.
BMP : Bone morphogenetic protein, DCC : detected in colorectal carcinoma, ECE : endothelin converting enzyme, ENCCs : enteric neural crest-derived cells, GDNF : glial cell-derived neurotrophic factor, GGF2 : glial growth factor 2, ND : not determined (Obermayr *et al.* 2013).

Transcription factor	Role in ENCCs	Phenotype of mouse ENS after perturbation	Evidence for role in Hirschsprung disease
Sox10	Progenitor maintenance and survival; regulates RET and Phox2b; promotes glial development[17,49,61,76,90,189,190]	*Sox10*$^{-/-}$: total gastrointestinal aganglionosis[17,191] *Sox10*$^{+/-}$: distal colonic aganglionosis[53,192]	Yes[189–191]
Sox8	Acts with Sox10, maintains progenitors[55]	*Sox8*$^{-/-}$ or *Sox8*$^{+/-}$: increases severity and penetrance of *Sox10*$^{+/-}$ phenotype[55]	ND
Foxd3	Regulates Sox10, maintains progenitors[196]	Foxd3$^{flox/-}$; Wnt1–Cre: lack of neurons in the entire gastrointestinal tract[196]	ND
Phox2b	Promotes survival, regulates RET expression[18,197,198]	*Phox2b*$^{-/-}$: lack of neurons in the entire gastrointestinal tract[18]	Yes[199]
Hand2	Promotes terminal differentiation of neuronal subpopulations[79,200]	*Hand2* cKO: reduction of neurons, loss of VIP and NOS neurons[71,200]	ND
Ascl1 (Mash1)	Promotes survival and development of neuronal subtypes[84]	*Mash1*$^{-/-}$: absence of neurons in oesophgus, reduction of 5-HT neurons in intestine[84]	ND
Pax3	Acts with Sox10 to activate RET expression[189]	*Pax3*$^{-/-}$: no ENCCs distal to the stomach *Pax3*$^{+/-}$: ENCC migration is delayed[189]	ND
AP-2 family	ap2α acts with foxd3 to regulate Sox10 in zebrafish[201]	ND	ND
Sox2	Expressed by ENS progenitors and then glia[202]	*Sox2*$^{-/-}$: mice die at preimplantation stages *Sox2*$^{+/-}$: no ENS defects reported[203]	Yes[204]
Zeb2 (Zfhz1b, SIP1)	Specification of vagal neural crest cells[205,206]	*Zeb2* cKO: colonic and partial small intestinal aganglionosis[206]	Yes[207–209]
Hlx	Promotes migration[210]	*Hlx*$^{-/-}$: neurons restricted to the stomach[210]	ND
TCF4	Interacts with the Wnt–β-catenin pathway	ND	Yes[211]
Hoxb5	Induces Ret expression, migration[212]	Antagonism of Hox5b: reduction of neurons or aganglionosis of the colon and ileum[212]	Yes[177]
HIPK2	Postnatal maintenance of neurons and glia[213]	*Hipk2*$^{-/-}$: postnatal increase in glia and progressive loss of neurons[213]	ND

Tableau 4 : Facteurs de transcription exprimés par les cellules de la crête neurale. AP-2 : activating protein 2, cKO : conditional knockout, HIPK2 : homeodomain interacting protein kinase 2, TCF4 : transcription factor 4, ZFHX1B : zinc finger E-box-binding homeobox 2 (Obermayr et al. 2013).

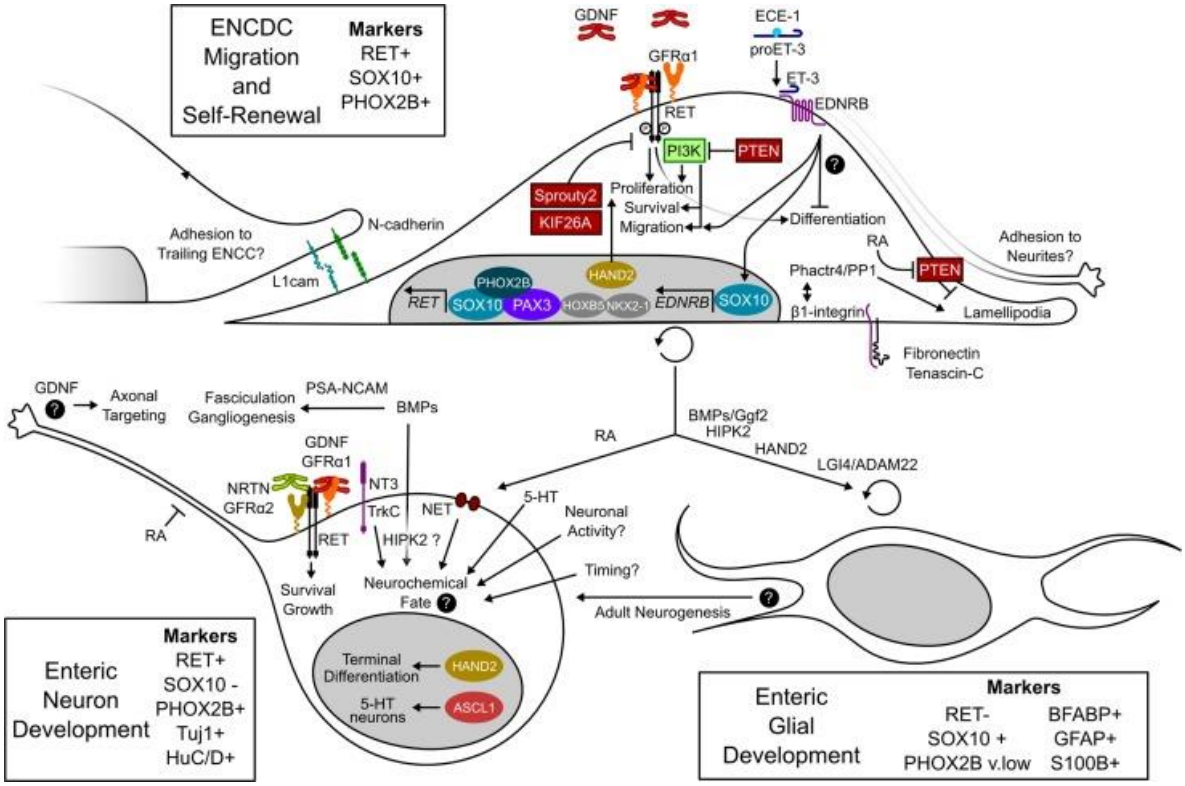


Figure 10 : voies de signalisation impliquées lors de la migration des cellules de la crête neurale.

Les voies de signalisation concernant les cellules dérivées de la crête neurale en migration sont illustrées sur la figure supérieure, celles de la différenciation neuronale en bas à gauche et celles de la différenciation gliale en bas à droite. Les marqueurs caractéristiques de ces cellules à ce stade du développement sont cités dans les encadrés correspondants. Les facteurs régulant l'activité de RET dans les cellules dérivées de la crête neurale sont indiqués en vert (activation) et rouge (inhibition). Les facteurs de transcription sont mis en évidence dans les noyaux (en gris si leur rôle n'est pas clairement défini). RA, retinoic acid; PSA-NCAM, polysialic acid-neural cell adhesion molecule; ECE, endothelin-converting enzyme; PP1, protein phosphatase 1; PTEN, phosphatase and tensin homolog; ENCDC, enteric neural crest-derived cell (Lake and Heuckeroth 2013).

La migration des CCN serait due également à des propriétés intrinsèques. Des CCN mises en culture avec des explants intestinaux de souris non colonisés ont migré dans le tube digestif dans les deux sens (Anderson *et al.* 2007). Afin de guider la colonisation, le mésenchyme exprimerait le GDNF selon un gradient spatio-temporel. Cette production différentielle permettant la migration rostro-caudale des CCN a été mise en évidence en hybridation *in situ* sur des embryons de souris entre E8.8 et E13.5 (Figure 11) (Natarajan *et al.* 2002).

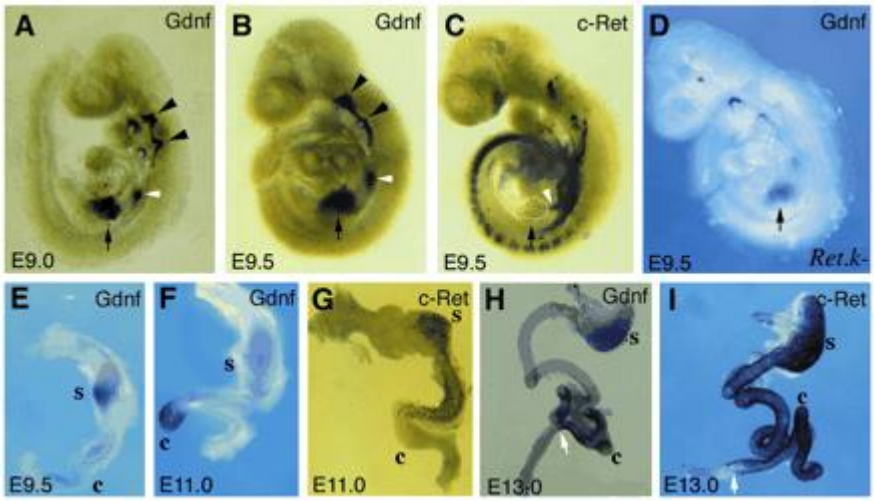


Figure 11 : Expression de GDNF et Ret chez l'embryon de souris.
Hybridation *in situ* d'embryon de souris sauvages (A-C), Ret.k- (D) et d'intestins de souris (E-I) ciblant les ARNm *Gdnf* (A,B,D,E,F,H) ou *Ret* (C,G,I). Sur les embryons de E9-9.5, les mRNA Gdnf sont detectés au niveau du mésenchyme splanchnique de l'estomac, des arcs branchiaux (flèches noires). Les cellules exprimant RET (C, flèches blanches) colonisent le territoire d'expression de GDNF.
GDNF est exprimé au regard de l'estomac (s) à E9.5 (E), du caecum (c) à E11 (F) puis du côlon à E13 (H) permettant une migration rostro-caudale des cellules exprimant RET (G,I) (Natarajan et al. 2002).

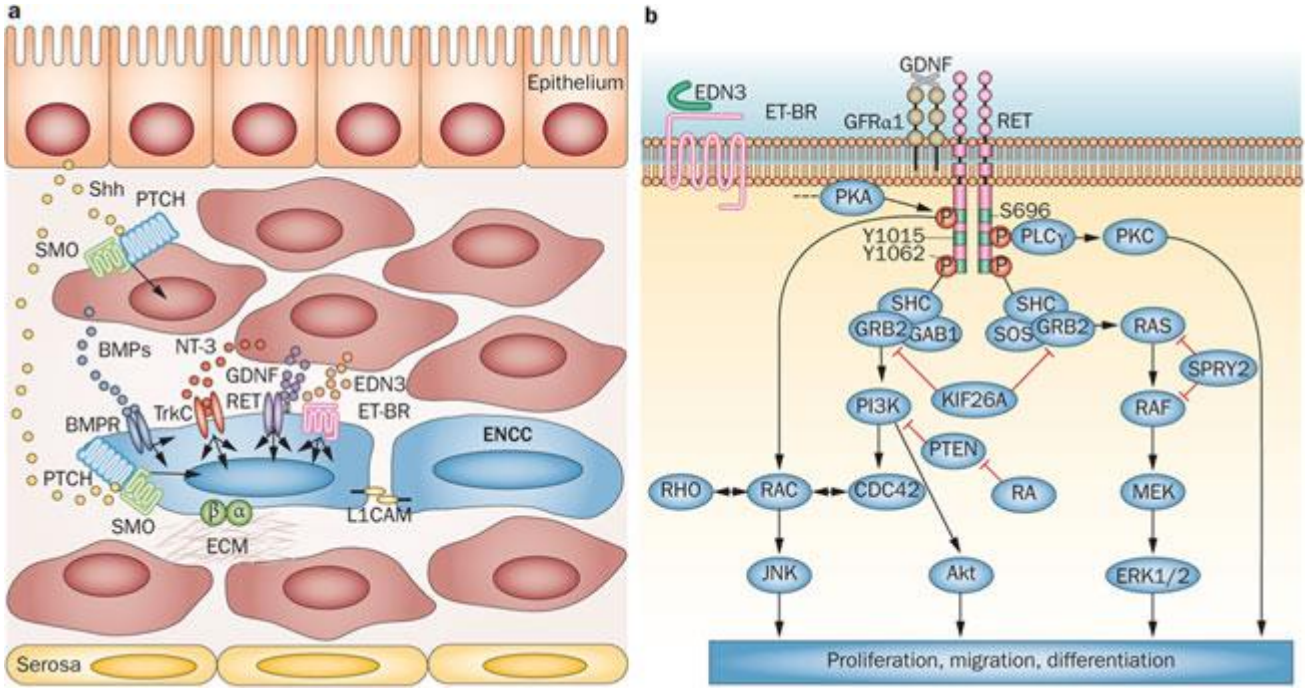


Figure 12 : Voies de signalisation influençant la survie, la prolifération, la migration et la différenciation des cellules dérivées de la crête neurale.
A : Les voies de signalisation principales impliquent le GDNF, EDN3, NT-3 et les BMP produits pas les cellules mésenchymateuses et agissent sur les récepteurs RET, ET-BR, TrkC et BMP (respectivement) présents sur les cellules dérivées de la crête neurale. Shh, par inhibition de PTCH, active SMO, agissant les cellules dérivées de la crête neurale, et permet l'expression de BMP par les cellules mésenchymateuses. Les intégrines régulent les interactions avec la matrice extracellulaire.

B : les voies de signalisation intracellulaires sont principalement activées par RET. L'activation de RET induit la phosphorylation de Y1015 et Y1062 qui nécessite GRB2 et PLCγ. RAC, RHO et CDC42 régulent la migration des cellules dérivées de la crête neurale. RHO et CDC42 régulent la proliferation. KIF26A, Sprouty2 (SPRY2) et PTEN sont des régulateurs négatifs de la voie RET. L'acide rétinoïque baisse le niveau d'expression de PTEN.

BMPs, bone morphogenetic proteins; ECM, extracellular matrix; EDN3, endothelin 3; ENCCs, enteric neural crest-derived cells; ENS, enteric nervous system; ET–BR, endothelin B receptor; GDNF, glial cell line-derived neurotrophic factor; GRB2, growth factor receptor-bound protein 2; KIF26A, kinesin-like protein KIF26A; NT-3, neurotrophin-3; PI3K, phosphoinositide 3-kinase; PTCH, patched; RA, retinoic acid; RET, proto-oncogene tyrosine-protein kinase receptor Ret; S, serine; Shh, Sonic hedgehog; SMO, smoothened; TrkC, NT-3 growth factor receptor; Y, tyrosine (Obermayr *et al.* 2013).

Par ailleurs, la migration caudale des CCN se déroule alors que l'intestin montre une croissance importante et des modifications tridimensionnelles de la position des anses digestives. Il a été montré récemment que certaines CCN coloniseraient le mésentère à E11.5 permettant de réaliser un court-circuit dans la migration et ainsi induire une colonisation plus précoce du côlon distal (Figure 13) (Nishiyama *et al.* 2012).

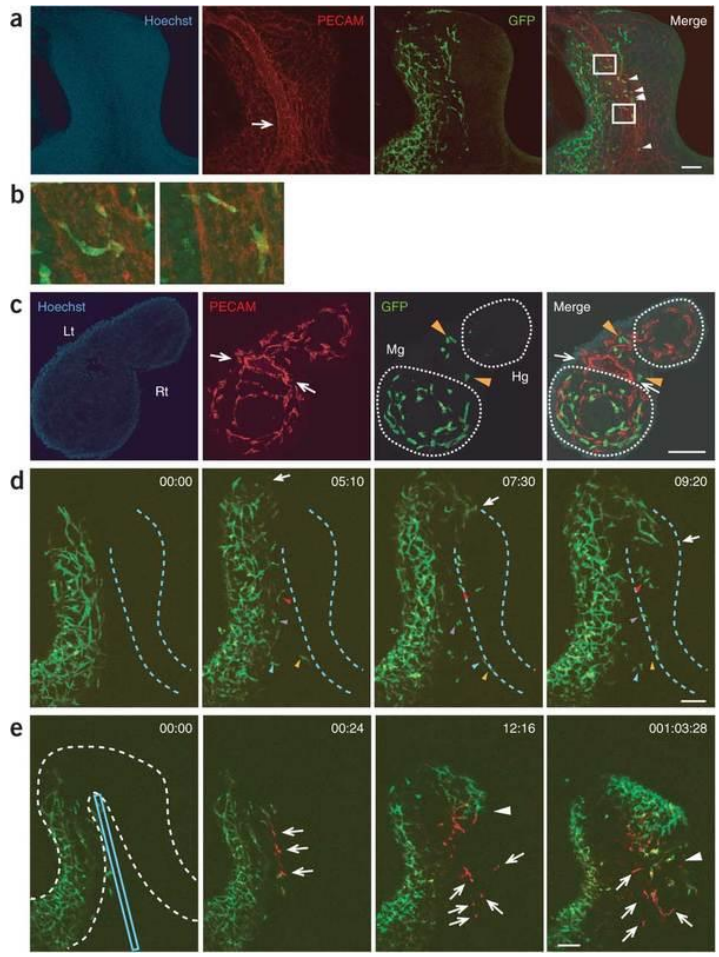


Figure 13 : Court-circuit mésentérique dans la migration des cellules de crête neurale.
Marquage Immuno-histochimique sur préparations whole-mount (A, B) et sections transversales (C) d'intestins d'embryons murins $Ret_{+/EGFP}$ à E11 permettant de visualiser en vert les cellules exprimant RET. Marquage nucléaire en bleu (Hoeschst) et vasculaire en rouge (PECAM). Les carrés blancs (A), agrandis (B), montrent des cellules de la crête neurale traversant les vaisseaux mésentériques. Sur les coupes transversales (C), ce court-circuit est montré par les triangles colorés en orange. Le front de migration des cellules de crête neurale est représenté (D) et par photoconversion (ligne bleue) les cellules qui effectuent le court-circuit sont représentées en rouge (E).
Les limites supposées du tube digestif sont indiquées en pointillé. Mg, midgut; Hg, hindgut; Lt, left, Rt, right. Echelle : 100µm (Nishiyama et al. 2012).

iii. Différenciation des cellules de la crête neurale

Pendant la colonisation, une partie des CCN stoppent leur migration. Les mécanismes responsables de cet arrêt de migration restent méconnus, mais la différenciation neuronale en serait le signal (Wu *et al.* 1999), bien que certains neurones continueraient de migrer quelques heures (Hao *et al.* 2009).

Les facteurs impliqués dans la neurogenèse seraient le GDNF (Sánchez *et al.* 1996), la neurturin (Doray *et al.* 1998), la neurotrophin-3 (NT-3) (Chalazonitis *et al.* 2001) et les bone morphogenetic proteins (BMPs) (Goldstein *et al.* 2005) qui sont produits par le mésenchyme. L'endothelin-3 (EDN3) par contre, inhiberait la différenciation neuronale (Leibl *et al.* 1999).

De plus, toutes les cellules dérivées de la crête neurale expriment Sox10. Quand ces cellules colonisent l'intestin, l'expression de Phox2B et Ascl1 et leurs transcrits (NB Phox et ASH-1) (Anderson, Stewart and Young 2006) supprimeraient l'expression de Sox10 dans les précurseurs neuronaux (Kim *et al.* 2003). La sur-expression de Sox10 a été reliée à une baisse de la neurogenèse (Nagashimada *et al.* 2012) et sa réduction d'expression à une différenciation prématurée (Okamura and Saga 2008). Par ailleurs, Hand2 induirait également une neurogenèse (D'Autréaux *et al.* 2007). Les voies de signalisation impliquées dans la différenciation des cellules dérivées de la crête neurale sont schématisées sur la figure 14.

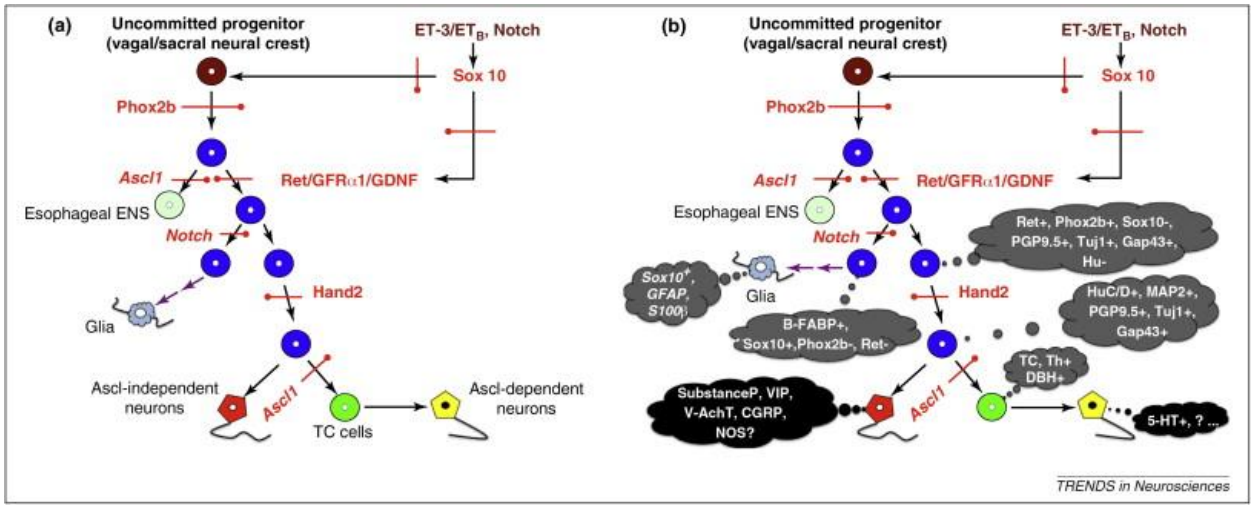


Figure 14 : voies de signalisation dans la différenciation des cellules dérivées de la crête neurale.

A : Le potentiel de différenciation des cellules dérivées de la crête neurale est progressivement restreint au cours du développement par l'expression de facteurs de transcriptions et de facteurs de croissance. Puis les facteurs de transcriptions sont supprimés (lignes rouges). En haut, les progéniteurs indifférenciés qui sortent des crêtes neurales expriment obligatoirement Sox10 et répondent à Notch et ET-3/ET_B qui les maintient en état indifférencié. Les cellules prolifèrent avant d'attendre l'intestin, puis le colonisent et expriment Phox2b. La formation de ganglions dans l'œsophage et l'estomac n'est pas sous la dépendance de l'activation de Ret par GDNF/GFRα (contrairement au reste du tube digestif) mais de Ascl1, responsable également de la différenciation neuronale (neurones catécholaminergiques et sérotoninergiques). En amont, Hand2 est aussi impliqué dans la différenciation neuronale. Les cellules gliales proviennent d'un précurseur commun par inhibition de Notch.

B : les gènes et/ou marqueurs identifiés dans les différentes étapes de différenciation sont identifiés dans les nuages. Les cellules engagées dans une différenciation neuronale expriment Ret, Phox2b, le marqueur neuronal PGP9.5 (protein gene product 9.5), Gap43 et TuJ1, n'expriment plus Sox10 et pas encore Hu (exprimé sous contrôle de Hand2). Les neurones dérivés de la voie Ascl1 dépendant sont catécholaminergiques (TH : tyrosine hydroxylase) et sérotoninergiques (5-HT) et les neurones Ascl1 indépendants expriment calcitonin gene related peptide (CGRP), substance P, vasoactive intestinal peptide (VIP), NO synthase (NOS) et le vesicular acetylcholine transporter (V-AchT). Les cellules engagées dans la lignée gliale expriment Sox10 et B-FABP et n'expriment plus Phox2b et Ret. Les cellules gliales exprimeront ensuite les marqueurs gliaux GFAP (glial fibrillary acidic protein) et S100β (Gershon 2010).

iv. Acquisition du phénotype neuronal

Le SNE présente une large diversité de neurones identifiés par leur phénotype neurochimique. Le délai d'expression de ces marqueurs neurochimiques est variable en fonction du marqueur et de la localisation (proximal/distal et plexus myentérique/plexus sous-muqueux).

L'essentiel des études portant sur la mise en place des caractéristiques neurochimiques du SNE a été réalisé chez le rongeur, mais les facteurs impliqués semblent être conservés entre les différentes espèces de mammifères (Grider 2003). Les marqueurs utilisés pour identifier l'ensemble des neurones sont appelés marqueurs pan-neuronaux et ne sont pas exprimés par les CCN en migration (Anderson, Stewart and Young 2006). Peu après la colonisation, dès E10 au niveau de l'estomac de souris, il a été décrit que près de 10 à 20% des cellules dérivées de la crête neurale exprimaient des marqueurs pan-neuronaux tels que Hu, neuron class III-tubulin (Tuj1), neurofilament-M et protein gene product 9.5 (PGP9.5) (Baetge and Gershon 1989; Young *et al.* 1999). Des cellules exprimant des marqueurs pan-neuronaux à proximité du front de migration des CCN chez l'embryon de souris ou de poulet ont été mises en évidence (Young *et al.* 1999; Young, Jones and McKeown 2002; Conner *et al.* 2003; Barlow *et al.* 2008), dont certaines continuaient de migrer caudalement (Hao *et al.* 2009) tout comme le font les neurones immatures dans le système nerveux central (Métin *et al.* 2008). Les marqueurs pan-neuronaux seraient présents 24 heures avant les marqueurs gliaux (Young, Bergner and Müller 2003).

La différenciation se poursuit, permettant l'apparition de sous-types neuronaux (Rothman and Gershon 1982; Epstein, Hudis and Dahl 1983; Matini, Mayer and Faussone-Pellegrini 1997). Chez l'embryon de souris, dès E11.5, ont été montrés des neurones exprimant la calbindin, la NOS (NO synthase), la Cart (cocaine and amphetamine regulated transcript) et certains neurites exprimant le canal potassique de conductance intermédiaire IKCa (Hao *et al.* 2009). Certains neurones matures montrent également un double marquage, par exemple VIP (vaso-intestinal peptide) et NOS. L'apparition de ce phénotype double est progressif, avec initialement un marquage NOS, puis quelques jours plus tard le marquage VIP (Rothman, Nilaver and Gershon 1984). Les neurones cholinergiques, marqués par la présence de 3H-acetylcholine ont pu être détectés chez la souris dès E10-E12 (Rothman and Gershon 1982), alors que les marquages par la choline acétyl-transferase (ChAT) ou par la vesicular acetylcholine transporter (VAChT) n'ont detecté ces neurones qu'à partir de E18.5. Plus récemment, Erickson *et al.* ont montré qu'à proximité du front de migration des cellules de la crête neurale, marquées par p75, certains neurones montraient un marquage contre la ChAT, soit vers E10.5 chez la souris (Figure 15). Les neurones cholinergiques ont ainsi été détectés au niveau de l'intestin grêle à E11.5, du côlon proximal à E13.5 et E16.5 pour le côlon distal. Chez le rat, les neurones ChAT-immuno-réactifs (IR) ont été détectés dans le plexus myentérique d'intestin grêle à partir de E18 (Vannucchi and Faussone-Pellegrini 1996). Le neuropeptide Y a été détecté chez la souris dès E13–13.5 (Branchek and Gershon 1989), la substance P à E14–14.5 (Rothman, Nilaver and Gershon 1984) et le calcitonin gene-related peptide (CGRP) (Furness *et al.* 2004), dès E17–17.5 (Branchek and Gershon 1989). Bergner *et al.* ont illustré la date de naissance de différents sous-type neuronaux en utilisant l'EdU (un analogue de thymidine) en période embryonnaire (Figure 16).

Chez le rat comme chez la souris d'autres sous-types neuronaux n'ont été détectés qu'après la naissance (Vannucchi and Faussone-Pellegrini 1996; Matini, Mayer and Faussone-Pellegrini 1997; Vannucchi, De Giorgio and Faussone-Pellegrini 1997; Young *et al.* 1998b).

Par ailleurs, il a été montré que l'apparition de ces marqueurs neuronaux apparaissait d'abord dans le plexus myentérique puis dans le plexus sous-muqueux (Pham, Gershon and Rothman 1991).

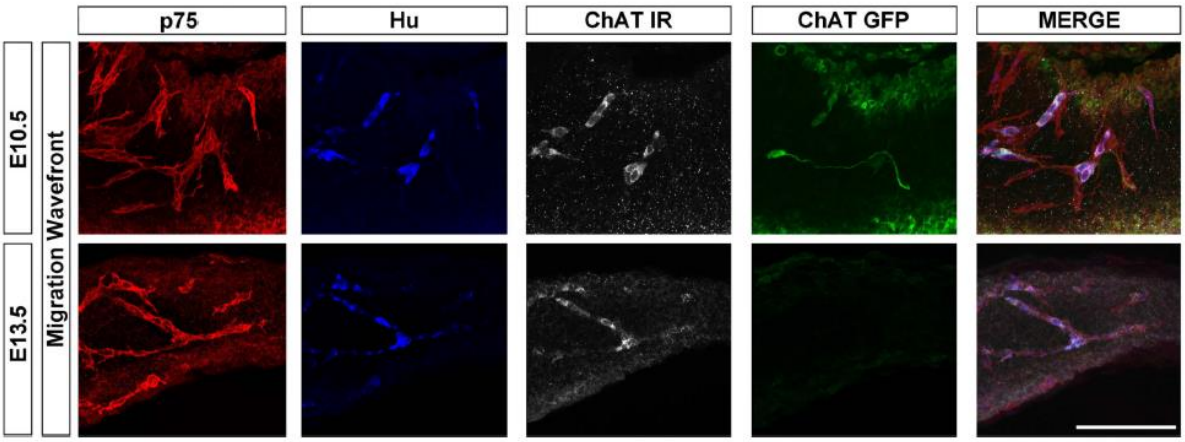


Figure 15 : Différenciation cholinergique des neurones issus des cellules de crête neurale.
Préparations « whole-mount » d'intestins de d'embryons de souris avec immuno-marquage ciblant les cellules dérivées de la crête neurales (p75), les neurones (Hu), les neurones cholinergiques (ChAT IR et ChAT GFP) à E10.5 et E 13.5. Echelle : 50µm (Erickson et al. 2014).

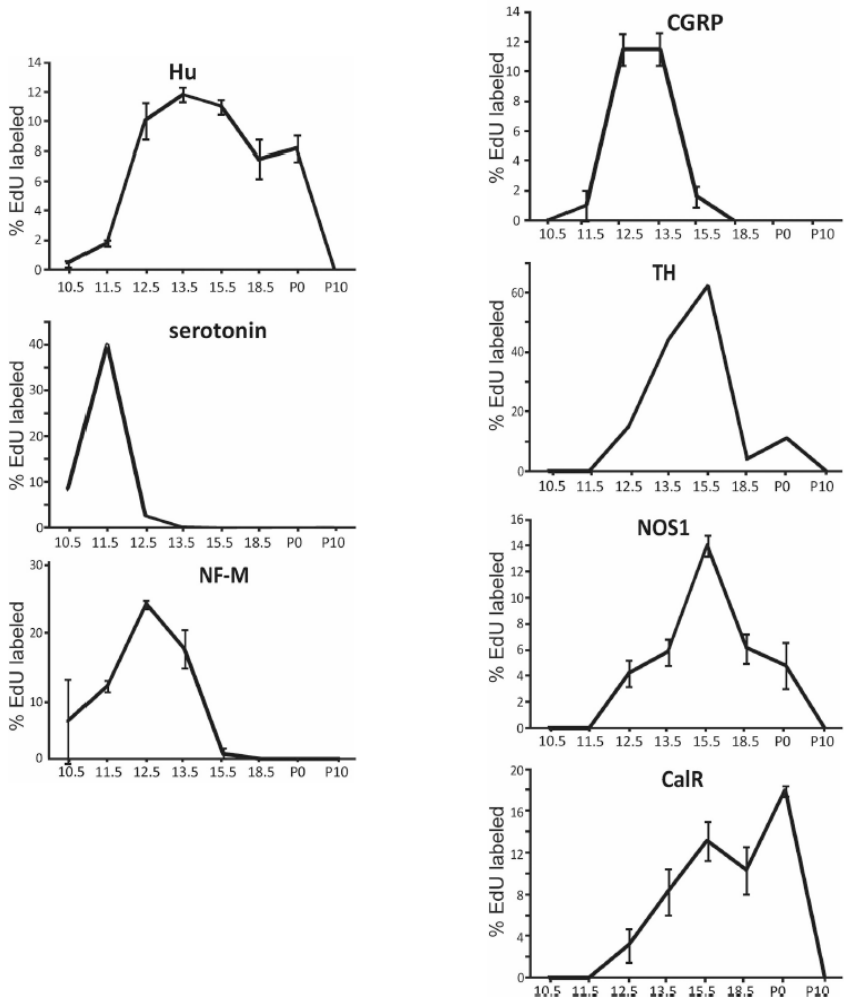


Figure 16 : Acquisition du phénotype neuronal au cours du développement.
Proportion des neurones myentériques d'intestins grêles de souris incorporant l'EdU injectés à différents âges. La date de naissance des sous-types neuronaux varient entre E10.5 et le 10ème jour de vie P10). Hu : marqueur pan-neuronal, NF-M : neurofilaments M, CGRP : calcitonin gene-related peptide, TH: tyrosine hydroxylase, NOS1 : NO synthase 1, CalR : calretinin (Bergner et al. 2014).

Les facteurs impliqués dans la différenciation dans un sous-type neuronal particulier sont encore peu connus. Néanmoins, il a été montré que ASCL1 induisait une différenciation sérotoninergique (Blaugrund *et al.* 1996), Hand 2 était impliqué dans la différenciation VIPergique et nitrergique (Lei and Howard 2011). Par ailleurs, RET influencerait le niveau d'expression de nNOS (Yan *et al.* 2004; Uesaka and Enomoto 2010; Wang *et al.* 2010). De même, le nombre de neurones CGRP a été diminué en cas de sous-expression de TrkC ou de son ligand NT3 (Chalazonitis *et al.* 2001).

v. Gliogenèse

L'autre contingent majeur des cellules dérivées de la crête neurale sont les cellules gliales entériques (CGE). Les premières cellules gliales ont été décrites peu après la différenciation neuronale, vers E11.5 chez la souris. Ces cellules se différencient à distance du front de migration des CCN et sont caractérisées par l'expression de BFABP (Young, Bergner and Müller 2003; Lozupone *et al.* 2013). Les facteurs de différenciation mis en jeu seraient le GDNF, les bone morphogenetic proteins (BMPs), et le neurotrophin-3 (NT3) (Heuckeroth *et al.* 1998; Chalazonitis *et al.* 2004).

Le GDNF à faible concentration a permis d'induire une différenciation neuronale et gliale alors qu'à forte concentration, des neurones seraient préférentiellement produits (Uesaka, Nagashimada and Enomoto 2013). Par ailleurs, Sonic hedgehog (Shh) inhiberait la neurogenèse induite par le GDNF au profit de la gliogenèse (Fu *et al.* 2004a).

Il a également été décrit que BMP4 induisait à la fois l'expression du tyrosine kinase receptor C (TrkC) favorisant la différenciation des neurones dopaminergiques TrkC-dependants (Chalazonitis *et al.* 2004) mais également la gliogenèse. L'hyper-expression de Noggin, antagoniste des BMPs supprimerait la différenciation gliale alors que l'activation des BMPs induirait une gliogenèse par expression d'ERB3 (Chalazonitis *et al.* 2011).

Par ailleurs, Sox10 inhibe la différenciation neuro-gliale et maintient un potentiel de cellules progénitrices (Bondurand *et al.* 2006). Sox2, exprimé dans l'intestin dès E10.5 (Heanue and Pachnis 2011) a permis également une différenciation gliale (Bondurand and Sham 2013). Enfin Notch inhiberait la neurogenèse en faveur d'une gliogenèse (Okamura and Saga 2008; Ngan *et al.* 2011). Le facteur de transcription Foxd3 exprimé par les cellules dérivées de la crête neurale présenterait aussi un rôle dans la différenciation neuronale, gliale, l'auto-renouvèlement des cellules de la crête neurale (Mundell and Labosky 2011; Mundell *et al.* 2012).

vi. Différenciation neuronale et gliale post-natale

La neurogenèse est précoce chez la souris, commence peu après la colonisation et se poursuit après la naissance. A la différence du système nerveux central, il ne semble pas persister à l'âge adulte de neurogenèse dans le SNE de façon basale mais uniquement sur stimulation comme montré après destruction des neurones myentériques par application séreuse de BAC (benzalkonium chloride) (Laranjeira *et al.* 2011). Une autre source de neurogenèse serait la glie : des cellules gliales se transformant en neurones chez la souris adulte (Laranjeira *et al.* 2011).

Un certain nombre de cellules dérivées de la crête neurale ont été isolées dans l'intestin en période post-natale chez le rongeur (Kruger *et al.* 2002; Bondurand *et al.* 2003) ainsi que chez l'homme (Rauch *et al.* 2006; Almond *et al.* 2007). Elles ont été mises en évidence soit par cytométrie de flux en ciblant des marqueurs spécifiques CD49b (integrin a2), p75NTR (Kruger *et al.* 2002; Joseph *et al.* 2011) ou par obtention de neurosphères en culture (Bondurand *et al.* 2003; Almond *et al.* 2007). Ces cellules se sont différenciées en neurones, en CGE et en myofibroblastes et, transplantées chez l'embryon de poulet ont montré un potentiel de migration (Kruger *et al.* 2002). Les cellules CD49b-positives expriment également p75NTR, GFAP, S100β, et Sox10.

Chez l'homme, des cellules progénitrices ont pu également être isolées et aboutir à des neurosphères à partir de fœtus, d'enfant de cinq ans (Rauch *et al.* 2006) et même chez l'adulte (Metzger *et al.* 2009; Hetz *et al.* 2014). Ces cellules transplantées dans des explants intestinaux de souris se sont différenciées en neurones et cellules gliales (Rauch *et al.* 2006; Almond *et al.* 2007; Lindley *et al.* 2008; Metzger *et al.* 2009; Hetz *et al.* 2014) et ont permis la restauration de la motricité digestive (Rauch *et al.* 2006; Lindley *et al.* 2008; Hetz *et al.* 2014).

En conditions physiologiques, la gliogenèse prédominerait sur la neurogenèse (Joseph *et al.* 2011). Par contre, en cas d'agression chimique (BAC), la neurogenèse prédomine (Laranjeira *et al.* 2011). Néanmoins il est difficile de dire si les CGE se transforment en neurones directement.

Une des voies pour obtenir une neurogenèse *ex vivo* sur plexus myentérique de souris adultes a été d'utiliser un agoniste des récepteurs sérotoninergiques de type 5-HT4 permettant l'activation de CREB (cAMP response element-binding protein)(Liu *et al.* 2009). Cela a également permis de réaliser des greffes neuronales sur des portions intestinales chez la souris (Metzger *et al.* 2009).

Dans les modèles murins d'agression gliale, des modifications phénotypiques des neurones myentériques ont été observées (neurones cholinergiques et nitrergiques) conduisant à des anomalies de motricité et de perméabilité intestinale (Aubé *et al.* 2006). Une des hypothèses serait le rôle neuro-protecteur des CGE, notamment par l'expression de la 15-Deoxy-D12,14-prostaglandinJ2 (15d-PGJ2) (Bach-Ngohou *et al.* 2010; Abdo *et al.* 2012).

vii. Formation de ganglions

Secondairement à la migration, les CCN forment des ganglions nerveux par agrégation cellulaire selon un gradient rostro-caudal. Le plexus myentérique apparait initialement dans l'intestin antérieur, puis dans l'intestin moyen et enfin dans l'intestin postérieur. Le plexus sous-muqueux se forme 2-3 jours après la formation du plexus myentérique et se forme par migration centripète de cellules du plexus myentérique (Fu *et al.* 2004b; Wallace and Burns 2005). Les facteurs impliqués dans la formation des ganglions sont les β1-integrin, les BMPs, Hand2 qui quand ils sont perturbés induisent désorganisation et modifications de taille ou de forme des ganglions. La densité neuronale au sein des ganglions nerveux de plexus sous-muqueux est plus faible que dans ceux du plexus myentérique (Wedel *et al.* 1999; Furness 2000).

viii. Autres modifications morpho-fonctionnelles

La paroi musculaire se développe avec un gradient de maturation rostro-caudal. Le muscle circulaire apparait à la 8ème semaine de développement au niveau de l'œsophage, à la 11ème semaine au niveau de l'intestin moyen. A la 14ème semaine, les couches musculaires sont visibles dans l'ensemble du tube digestif (Fu *et al.* 2004b).

Les cellules interstitielles de Cajal (CIC) sont aussi d'origine mésodermique (Young *et al.* 1996). Elles sont identifiées par le marquage c-kit (Figure 17), récepteur tyrosine kinase qui est essentiel dans leur développement et leur fonctionnement (Maeda *et al.* 1992). Ces cellules ne semblent pas avoir de gradient rostro-caudal d'apparition ou de maturation. Elles apparaissent dans l'intestin moyen à partir des 7-9ème semaines et prennent un aspect mature vers les 12-14èmesemaines de développement (Fu *et al.* 2004b; Wallace and Burns 2005).

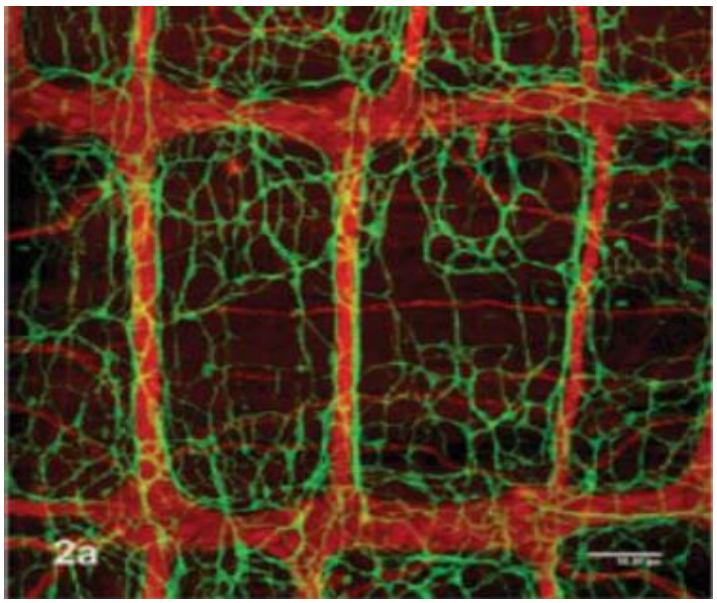


Figure 17 : Les cellules de Cajal.
Microphotographie de plexus myentérique de cobaye avec immuno-marquage des neurones (PGP, rouge) et des cellules de Cajal (kit, vert). Echelle 80 µm (Komuro 2006).

3- Contrôle des fonctions digestives par le système nerveux entérique

a. Contrôle de la motricité digestive

i. Sur un système mature

La motricité digestive peut se schématiser en trois phénomènes moteurs sous contrôle du SNE : la segmentation qui consiste en une activité musculaire statique de contraction et relaxation permettant de mixer le bol alimentaire et de l'exposer aux enzymes digestives. Le péristaltisme permet l'avancée du bol alimentaire dans un sens oro-anal. Enfin, les complexes moteurs migrants (CMM) sont des contractions digestives intenses, lentes et rythmées survenant en dehors des repas qui permettent le nettoyage des débris non digestibles, du mucus et des débris cellulaires.

Le péristaltisme intestinal est un exemple concret du contrôle des fonctions digestives par le SNE. En effet l'intestin explanté d'un organisme et donc déconnecté de toute afférence nerveuse extrinsèque (Langley and Magnus 1905), présente des contractions régulières permettant la progression du bol alimentaire dans le sens oro-anal (Bayliss and Starling 1900). Les mécanismes impliqués dans le péristaltisme sont décomposés ainsi (Figure 18) : les cellules entérochromaffines de l'épithélium digestif libèrent de la sérotonine en réponse à une stimulation mécanique (bol alimentaire) ou chimique (présence d'acides gras, de carbohydrates, d'acidité, ou changement d'osmolarité). La sérotonine stimule les IPAN (récepteurs 5-HT1, 5-HT3, 5-HT4) (Furness *et al.* 1998) qui sont en connexion avec des interneurones par l'intermédiaire de la substance P, de l'acétylcholine, du CGRP.

En amont, les interneurones ascendants sont immunoréactifs pour la ChAT (choline acetyl transferase), la substance P, et la calrétinine (Brookes *et al.* 1997). La transmission entre interneurones est médiée par l'acétylcholine (Ach) qui se fixe sur les récepteurs nicotiniques (nAChR). La transmission entre les interneurones et les neurones moteurs excitateurs est médiée par l'ACh et les tachykinines (récepteurs NK3)(Bornstein, Costa and Grider 2004). Ces neurones moteurs libèrent l'ACh et les tachykinines, notamment la Substance P, la neurokinine A et l'enképhaline, (Holzer, Schluet and Maggi 1993; Lippi *et al.* 1998; Furness *et al.* 2003) et entraînent une dépolarisation et une contraction du muscle

intestinal. Cette activité est bloquée par les antagonistes des récepteurs muscariniques (atropine) et aux tachykinines.

En aval, les interneurones descendants sont en relation avec les neurones moteurs inhibiteurs. Trois groupes d'interneurones descendants ont été décrits en fonction de leur immuno-réactivité : ChAT/NOS, ChAT/5-HT et ChAT/SOM (somatostatine). Ce dernier est plutôt associé dans la coordination de complexes moteurs migrants (Furness 2000). La transmission synaptique vers les neurones inhibiteurs est assurée essentiellement par l'ACh et par l'adénosine triphosphate (ATP) via les récepteurs P2X. Les neurones moteurs inhibiteurs, contiennent de l'ACh, et du VIP, leurs médiateurs sont l'ATP, le NO ou le VIP (Furness 2000). L'activation des neurones moteurs inhibiteurs induit une hyperpolarisation et la relaxation du muscle lisse intestinal. Une réponse musculaire rapide est obtenue par l'ATP, ou le NO. La réponse musculaire est retardée si elle est médiée par le VIP (Hansen 2003). Le NO est produit par la NO-synthase (NOS), dont l'activité est bloquée par le N(G)-nitro-L-Arginine-Méthyl Ester (L-NAME).

Au final, la coordination entre contraction d'amont (majoritairement sous contrôle de l'acétylcholine et des tachykinines) et une relaxation d'aval (médiée par le NO, le VIP et l'ATP) permet l'avancée du bol alimentaire.

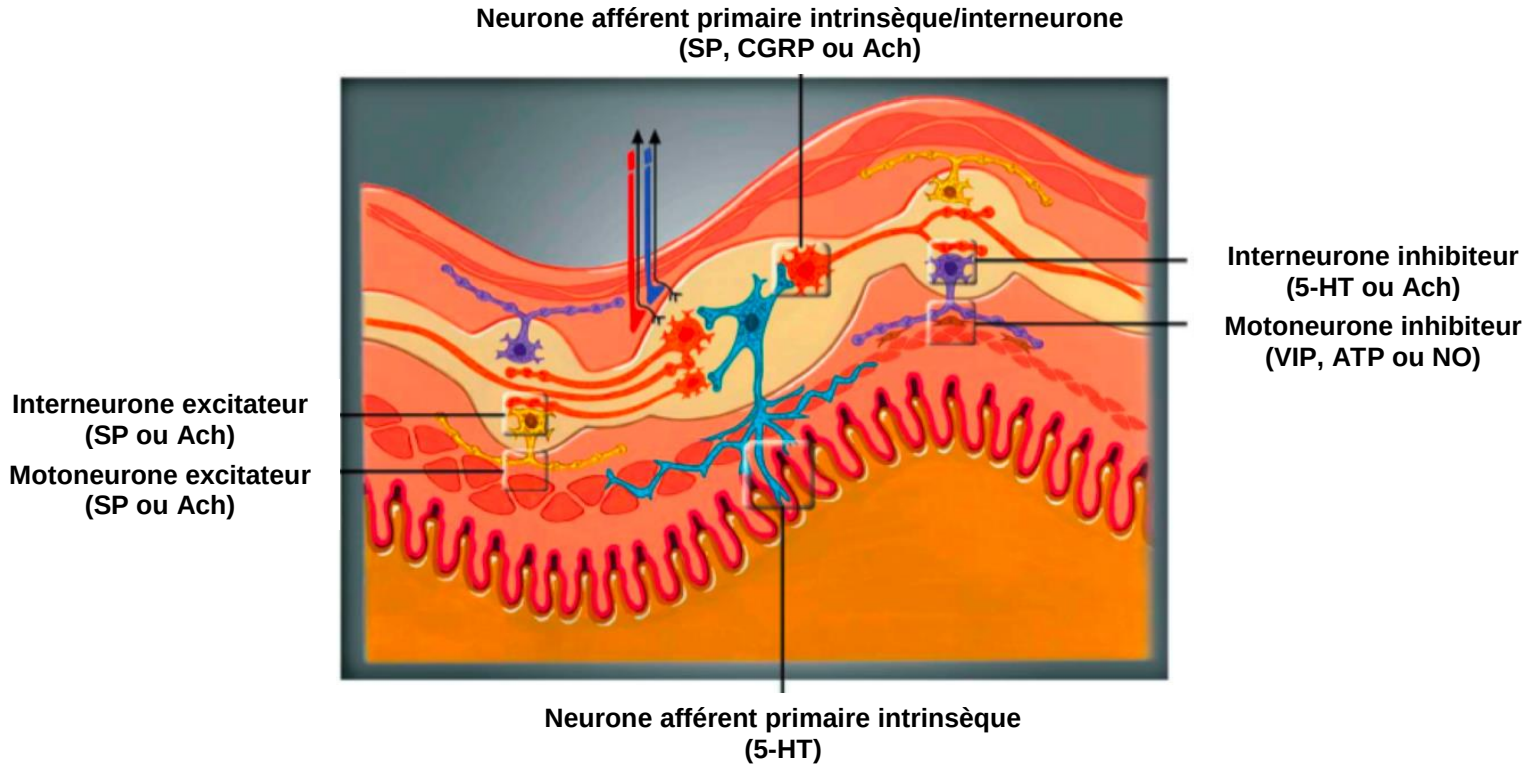


Figure 18 : Schéma du réflexe péristaltique dans l'intestin grêle.
Un stimulus luminal (mécanique ou chimique) induit une libération de sérotonine (5-HT) par les cellules entérochomaffines qui activent les IPAN connectés aux interneurones ascendants excitateurs et descendants inhibiteurs. Les interneurones excitateurs libèrent de l'acétylcholine (Ach) et de la substance P (SP) pour exciter les motoneurones qui libèrent de l'Ach et de la SP au niveau du muscle qui présentera une contraction. Les motoneurones inhibiteurs sont activés par la 5-HT et l'Ach pour libérer du monoxyde d'azote (NO), du vaso-intestinal peptide (VIP) ou de l'adénosine triphosphate (ATP) vers le muscle qui présentera une relaxation. D'après (Hansen 2003).

ii. Maturation de la motricité digestive chez l'homme

Chez l'homme, le fœtus à terme absorberait 450 mL de liquide amniotique par jour (Pritchard 1966) (Tableau 5). La déglutition efficace sans fausse route est observée à partir de 32 semaines de grossesse impliquant l'utilisation de sondes pour la nutrition des prématurés (Lau, Smith and Schanler 2003). Concernant la motricité œsophagienne, les contractions observées chez le prématuré sont plus courtes et moins rapides que chez l'enfant à terme (Jadcherla 2002).

TABLE 2. AMNIOTIC FLUID SWALLOWING BY IMMATURE FETUSES

Fetal weight (gm.)	*Time of labeling (hr. before delivery)*	*Volume swallowed (ml.)*		*Total volume (ml.)*	
		Total	*Per 24 hr.*	*Measured*	*Isotope dilution*
135	24	7	7	305	290
330	24	16	16	—	320
498	29	30	25	290	300
550	23	72	76	610	—
1240	22	110	120	—	575

Tableau 5 : Evaluation isotopique de la déglutition prénatale chez l'homme.
Le volume de liquide amniotique dégluti par le fœtus a été évalué par mesure isotopique à différents âges gestationnel. Il a été observé une augmentation progressive du volume dégluti en fonction du terme gestationnel (Pritchard 1966).

La demi clairance gastrique a été estimée à environ 30 minutes entre 25 et 36 SA par méthode échographique (évolution de la surface sectionnelle de l'antre en regard de l'aorte -ACSA antral cross sectional area-) (Newell, Chapman and Booth 1993). La vidange gastrique (méthode de dilution) représente environ un volume de 20 à 30 mL/heure/0.1m^2 de surface corporelle chez le nouveau-né (Cavell 1981). Les facteurs modulant la vidange gastrique seraient l'âge gestationnel (Figure 19), le poids de naissance, le type d'alimentation (lait maternel ou artificiel) sur l'évaluation par test respiratoire au lait marqué au ^{13}C-octanoic acid de Ramirez *et al*.

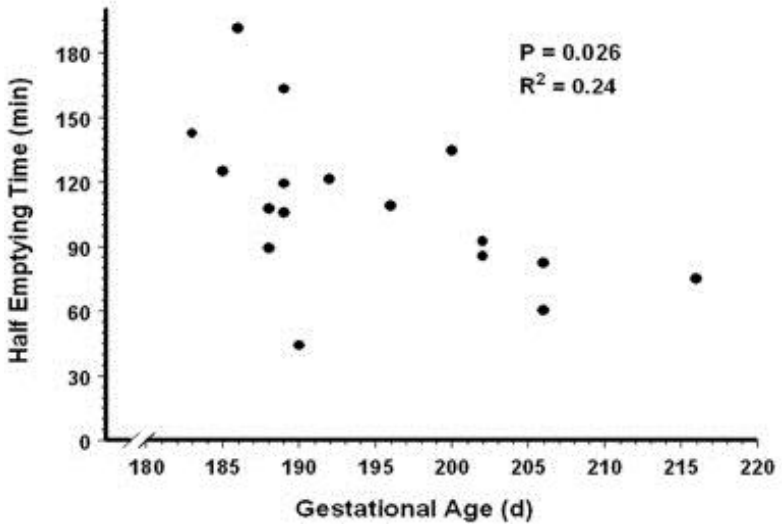


Figure 19 : Evaluation de la vidange gastrique en fonction du terme gestationnel.
La vidange gastrique a été évaluée par test respiratoire au 13C-octanoic acid incorporé dans le lait et mesuré dans l'air expiré. Les auteurs ont observés une décroissance du temps de vidange gastrique en fonction de l'âge gestationnel croissant (Ramirez, Wong and Shulman 2006).

La coordination antro-duodénale a été mesurée par manométrie en utilisant des capteurs de pressions raccordés à une sonde d'alimentation. Alors que les enfants à terme présentent quasi-systématiquement une contraction antrale suivie d'une contraction duodénale, les prématurés présentent cette coordination de manière aléatoire. Le nombre d'événement coordonnés augmente avec l'âge et l'avancée du terme de l'enfant (Ittmann, Amarnath and Berseth 1992).

La motricité de l'intestin grêle a également été étudiée par manométrie (Berseth 1989). Cette étude a montré que les clusters d'activité motrice évoluaient avec l'âge gestationnel. Avec le terme, les clusters de contractions sont moins fréquents, plus amples et prolongés, permettant de calculer un index de motricité intestinale (Figure 20).

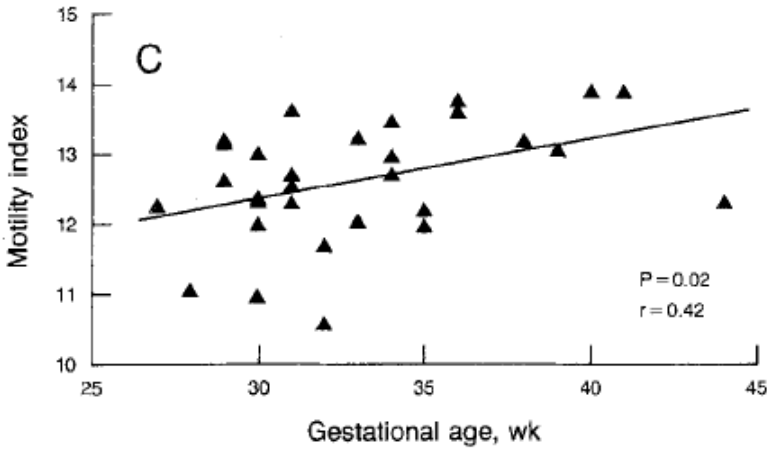


Figure 20 : Index de motricité intestinale proposé par Berseth *et al*. sur étude manométrique.
L'index de motricité intestinale a été calculé en par $\log_e$ (somme des amplitudes x nb de contractions). Cette étude a montré une augmentation de l'index de motricité intestinale avec l'avancée de l'âge gestationnel (Berseth 1989).

Le temps de transit gastro-anal varie entre 8 et 96 heures chez le prématuré comparé à 4 à 12 heures chez l'enfant à terme. L'activité propulsive du côlon semble quant à elle inhibée jusqu'à la naissance évitant la diffusion du méconium dans le liquide amniotique. Chez l'enfant à terme, l'issue du méconium est observée dans les premières 24 heures de vie, et est retardée chez le prématuré associée à une durée d'élimination complète plus longue (Figure 21) (Bekkali *et al.* 2008).

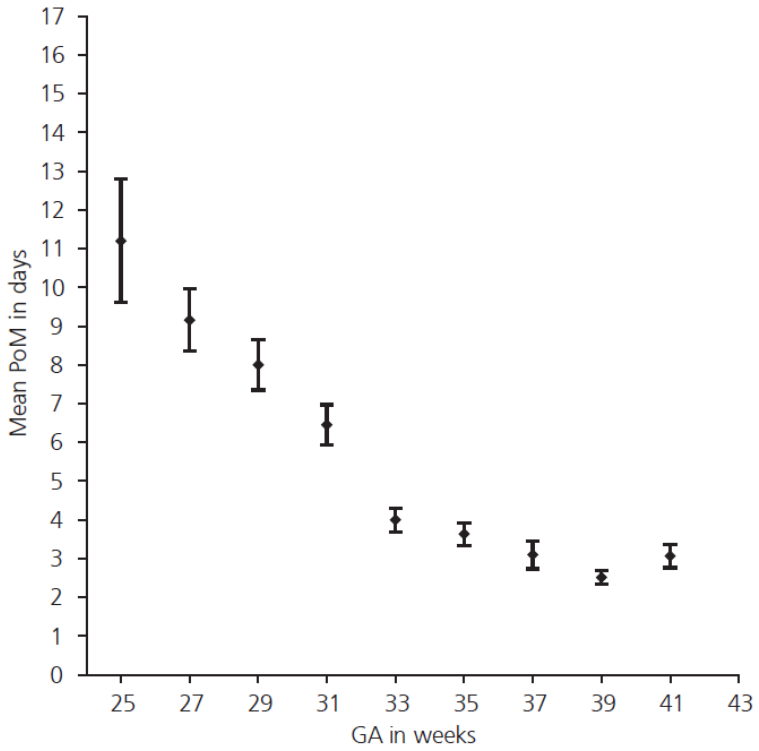


Figure 21 : Corrélation entre âge gestationnel et la durée d'émission du méconium. PoM : Passage of meconium, GA : gestational age. Cette étude a montré que la durée d'expulsion du meconium diminuait significativement en fonction du terme de naissance (Bekkali et al. 2008).

iii. Maturation du contrôle de la motricité digestive par le SNE : exemples murins

Chez la souris, des contractions duodénales spontanées ont été montrées se propageant dans le sens oro-anal et inverse dès E14.5, soit approximativement six jours avant la naissance (Burns *et al.* 2009). Ces contractions semblent indépendantes des cellules interstitielles de Cajal (CIC), apparaissant après dans le développement et du contrôle neuronal car elles persistent après blocage de l'activité neuronale par tétrodotoxine (TTX) (Burns *et al.* 2009). Dans le jéjunum, des contractions ont également été constatées entre E14.5 et E16.5, indépendamment du contrôle des CIC ou des neurones (Figure 22) (Torihashi, Ward and Sanders 1997; Ward *et al.* 1997). Vers E18.5 (2 jours avant la naissance), les contractions observées sont modifiées par la TTX démontrant l'apparition du contrôle neuronal (Burns *et al.* 2009). Au niveau du côlon, des contractions spontanées ont été constatées à E11.5, avant même la colonisation des CCN (Lindley *et al.* 2008). Plus tard, chez des souris dépourvues de neurones entériques dans le côlon, à E18.5, des contractions coliques ont également été mises en évidence (Anderson *et al.* 2004). Ces éléments montrent que le SNE n'est pas responsable de l'activité motrice propulsive chez le fœtus de souris contrairement aux CIC dont l'inhibition du récepteur kit aboli les contractions prénatales du côlon (Lindley *et al.* 2008). A la naissance, les contractions coliques restent indépendantes de l'activité neuronale car inchangées par la TTX (Roberts *et al.* 2007) sauf au niveau du côlon distal où elles deviennent plus fréquentes et plus irrégulières (Lindley *et al.* 2008) suggérant que l'activité neuronale a initialement plutôt un rôle modulateur que générateur dans cette période périnatale. Chez la souris adulte, les complexes moteurs migrants (CMM) du côlon sont contrôlés par l'activité neuronale. Les CMM coliques ne sont pas détectables au 4ème jour de vie mais au 6ème en utilisant un inhibiteur du NO (Roberts *et al.* 2007). Les CMM maturent rapidement et présentent les mêmes propriétés électrophysiologiques que les souris adultes dès le 10ème jour de vie (Roberts *et al.* 2007).

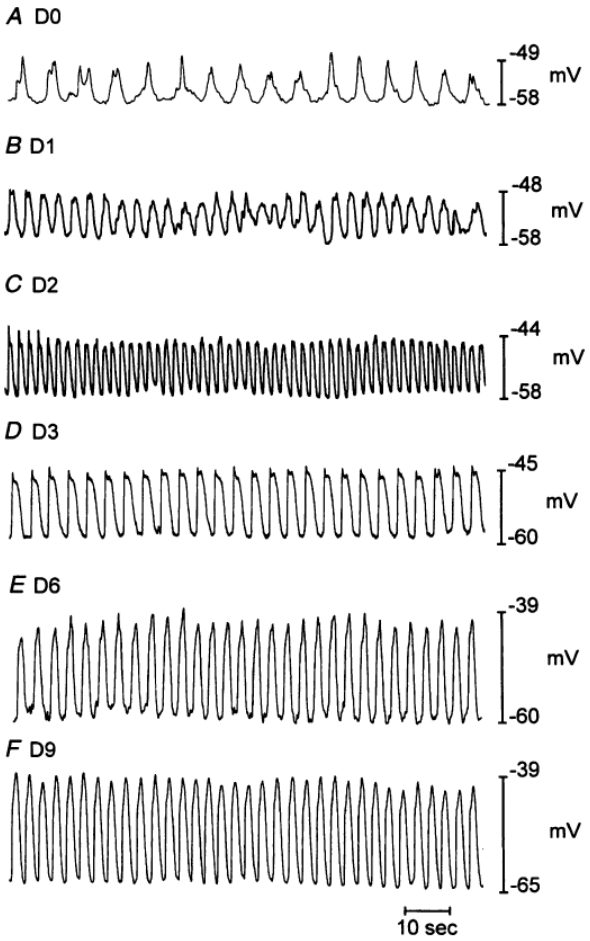


Figure 22 : Mise en place postnatale de l'activité motrice du jéjunum de souris.
La fréquence, l'amplitude et la durée des contraction jéjunales évoluent progressivement vers une motricité mature entre la naissance (D0) et le 9ème jour de vie (D9) chez la souris (Ward et al. 1997).

b. Contrôle de la perméabilité intestinale par le SNE

i. Sur un système mature

Le rôle du SNE sur le maintient de l'intégrité de la barrière épithélio-intestinale (BEI) implique plusieurs modes d'actions : rôle sur la perméabilité, sur la prolifération et la différenciation cellulaire, sur la protection et la réparation de la barrière (Figure 23).

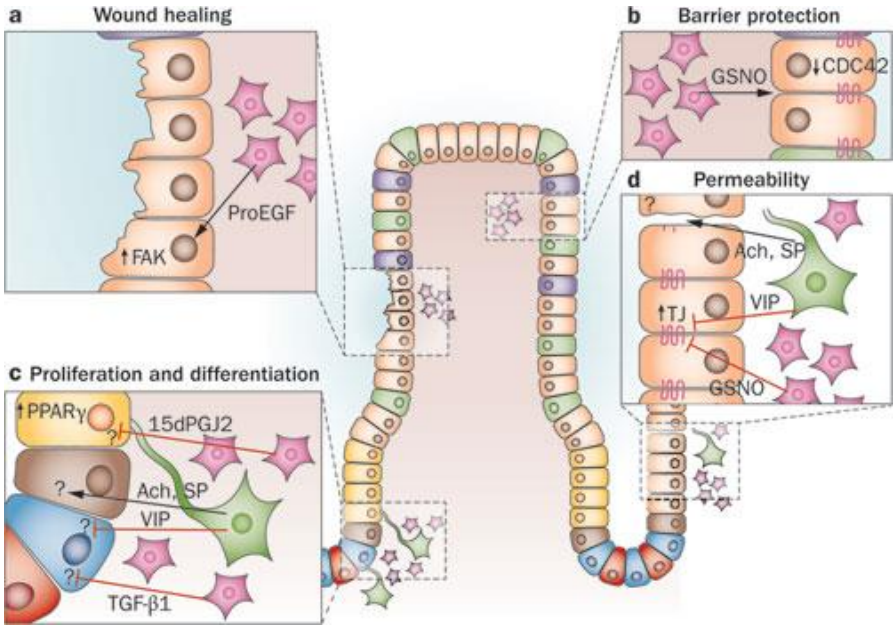


Figure 23 : Implication des composants du système nerveux entérique sur l'intégrité de la barrière épithélio-intestinale.
Les neurones entériques (vert) et les cellules gliales (rose) produisent des facteurs solubles régulant la prolifération, la différenciation, la perméabilité des entérocytes (marron), cellules souches intestinales (bleu), cellules de Paneth (rouge), cellules entéroendocrines (violet) et les cellules de Goblet (vert clair). A: cicatrisation: les cellules gliales entériques accélèrent la cicatrisation de lésions de la barrière par l'expression de pro-EGF induisant une augmentation de l'expression et l'activité de FAK. B : protection de la barrière : les lésions induites par un pathogène (*Shigella flexneri*) induisent la sécrétion de GSNO par les cellules gliales entériques conduisant à une baisse d'expression de CDC42 et une augmentation de la résistance de la barrière. C: prolifération et différenciation: les neurones et les cellules gliales sécrètent des médiateurs qui limitent (VIP, TGF-β1 et 15dPGJ2) ou qui augmentent (Ach, SP) la prolifération cellulaire. D: perméabilité: la perméabilité de la barrière épithélio-intestinale peut être modifiée par des médiateurs neuro-gliaux, augmentée par l'Ach, réduite par VIP et GSNO en augmentant l'expression de ZO-1. Abréviations : Ach, acétycholine; ENS, enteric nervous system; FAK, focal adhesion kinase; GSNO, S-nitrosoglutathione; IEB, intestinal epithelial barrier; PPARγ, peroxisome proliferator-activated receptor γ; SP, substance P; TJ, tight junction proteins; VIP, vasoactive intestinal peptide (Neunlist *et al.* 2013).

La perméabilité intestinale permet le passage de molécules situées dans la lumière digestive (eau, électrolytes, nutriments,…) vers la circulation systémique tout en évitant les agents pathogènes (bactéries, toxines, toxiques). Ce filtre actif et sélectif est le fait de la (BEI). La cohésion de l'épithélium intestinal est un facteur majeur de l'intégrité de cette barrière qui est sous la dépendance de complexes multiprotéiques : les jonctions serrées (tight junctions), les jonctions adhérentes et les desmosomes (Figure 24).

Les jonctions serrées se trouvent au pôle apical des cellules épithéliales et sont composées d'une plaque protéique connectée au cytosquelette d'actine et de protéines transmembranaires. Ces dernières sont constituées de claudine, d'occludine et de la protéine JAM et sont également reliées au cytosquelette par les protéines Zonula Occludens (ZO-1, ZO-2 et ZO-3) et la cinguline (Förster 2008). Les jonctions serrées réalisent alors des pores dont la taille permet le passage para-cellulaire de molécules (Tsukita, Furuse and Itoh 2001). Les jonctions adhérentes connectent les cellules épithéliales par la E-cadhérine qui est reliée au cytosquelette par les protéines α-, γ-, β- et p120 caténines, l'α-actinine et la vinculine (Ebnet 2008). Les desmosomes relient les cellules entre elles par des filaments de cytokératine et permettent une forte cohésion cellulaire (Garrod, Merritt and Nie 2002). Par ailleurs, les jonctions communicantes (gap junctions) réalisent des canaux de communication entre les cellules en permettant le passage de signaux chimiques ou électriques en fonction de l'état de phosphorylation des connexines (Prochnow and Dermietzel 2008).

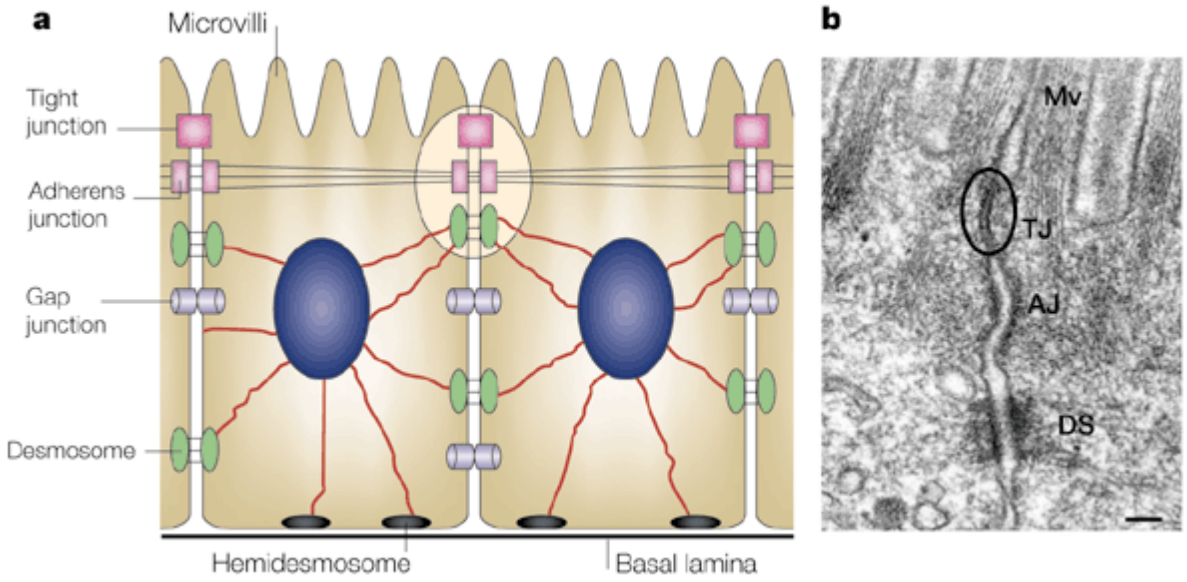


Figure 24 : Le complexe jonctionnel des cellules épithéliales intestinales.
Représentation schématique du complexe jonctionnel situé sur la partie apicale des faces latérales de la cellule épithéliale (A) et correspondance en microscopie électronique de cellules épithéliales murine (B). Le complexe jonctionnel est constitué de jonctions serrées (TJ, tight junctions), de jonctions adhérantes (AJ, adherans junctions), de jonctions communicantes (gap junctions). Echelle: 200 nm.(Tsukita, Furuse and Itoh 2001).

L'étude du rôle du SNE sur le contrôle de la perméabilité intestinale est plus récente que pour le contrôle de la motricité. L'activité neuronale serait responsable d'une augmentation de la perméabilité para-cellulaire car l'utilisation de TTX permet d'éviter l'augmentation de perméabilité induite par l'allergie à l'ovalbumine chez le rat (Crowe *et al.* 1993) ou par le jeûne du porcelet (flux d'inuline)(Hayden and Carey 2000). L'utilisation d'antagonistes des récepteurs cholinergiques montre également les mêmes résultats chez ce dernier. Dans un modèle de co-culture contenant de la sous-muqueuse et une monocouche de colonocytes HT-29, il a été montré que la stimulation électrique de neurones sous-muqueux induisait une baisse de la perméabilité para-cellulaire (flux de Dextran-FITC), une augmentation de l'expression de ZO-1 dans les cellules épithéliales. Cet effet était inhibé par l'utilisation de TTX ou d'antagoniste du VIP (Neunlist *et al.* 2003). D'autres études ont montré un effet direct de neuromédiateurs sur la perméabilité de muqueuses coliques. Le

VIP diminue la perméabilité, l'acétylcholine l'augmente (Blais, Aymard and Lacour 1997). Par ailleurs, la neurokinine A augmente la perméabilité duodénale chez le rat, corrigée par le VIP (Hällgren, Flemström and Nylander 1998).

Les cellules gliales entériques ont également un rôle dans la régulation de l'homéostasie de la BEI. Par analogie, les astrocytes du SNC contrôlent l'adhésion des cellules endothéliales des vaisseaux cérébraux formant la barrière hémato-encéphalique (Abbott, Rönnbäck and Hansson 2006), les CGE, contrôlant la BEI (Savidge *et al.* 2007). Le premier constat est histologique : les CGE muqueuses entretiennent des rapports étroits avec les cellules épithéliales. Les prolongements gliaux entrent en contact avec la membrane basale de l'épithélium et les capillaires de la lamina propria.

Les CGE contrôlent la prolifération et l'adhésion des cellules épithéliales, notamment par la sécrétion de TGF β1 (Transforming growth factor beta 1). Ce facteur diminue la prolifération épithéliale et augmente la surface apicale des cellules (Neunlist *et al.* 2007). Par ailleurs, la suppression conditionnelle des cellules GFAP (et des cellules gliales) dans le tube digestif de souris transgéniques induit une augmentation de la perméabilité digestive et une inflammation jéjuno-iléale intense (Bush *et al.* 1998). Cet effet est également retrouvé par l'inhibition de production du S-nitrosoglutathione (GSNO) par inhibition de l'expression des F-actin et des protéines associées aux jonctions serrées. *In vitro*, le GSNO inhibe l'action des tumor necrosis factor alpha (TNF α), interferon gamma (INFγ), et interleukin 1 beta (IL1 β) (Savidge *et al.* 2007; Cheadle *et al.* 2013) et protège la barrière de pathogènes comme Shigella flexneri, une bactérie entéro-invasive (Flamant *et al.* 2011). Les CGE ont également un rôle dans la vitesse de réparation de la BEI (Van Landeghem *et al.* 2011) notamment par la production de pro-epidermal growth factor (pro-EGF) et de la voie des focal adhesion kinase (FAK).

ii. Evolution de la perméabilité intestinale chez l'homme

La perméabilité intestinale a été évaluée chez le nouveau-né par des tests d'absorption de sucres. Ces tests consistent à mesurer l'excrétion urinaire de sucres de différentes tailles. Par exemple, le lactulose et le mannitol ou le l-rhamnose sont utilisés pour leurs tailles différentes, respectivement. En conditions physiologiques, le rapport L/M dans les urines est faible. Si le rapport est élevé, cela peut être soit le fait d'une augmentation de la perméabilité aux macromolécules, soit une baisse de perméabilité au mannitol. Cette technique a été utilisée chez le nouveau-né, en particulier chez le prématuré.

La perméabilité intestinale semble augmenter dans la première semaine de vie, notamment chez les nouveaux nés prématurés avant 28 semaines, puis diminue au cours de la deuxième semaine de vie. A la naissance, la perméabilité intestinale des prématurés (26-36 semaines de grossesse) est plus élevée que chez les enfants à terme (van Elburg *et al.* 2003). Cette différence n'est plus retrouvée 3 ou 6 jours plus tard. Par ailleurs, dans les deux premiers jours de vie, la perméabilité n'est pas corrélée au terme ni au poids de l'enfant (van Elburg *et al.* 2003).

La perméabilité intestinale semble être modifiée par la nutrition. Elle diminue avec la nutrition entérale (Shulman *et al.* 1998; Rouwet *et al.* 2002) et en l'absence de nutrition entérale, les enfants nés avant 28 semaines gardent au 7ème jour de vie une perméabilité intestinale supérieure aux enfants nés à terme (Figure 25) (Rouwet *et al.* 2002). Il a également été montré que la nutrition par lait artificiel induisait une perméabilité plus importante au 28ème jour de vie par rapport à l'alimentation par lait de mère. Par contre, l'administration prénatale de corticoïdes (à visée pulmonaire) décroit le perméabilité intestinale au 28ème jour de vie (Shulman *et al.* 1998).

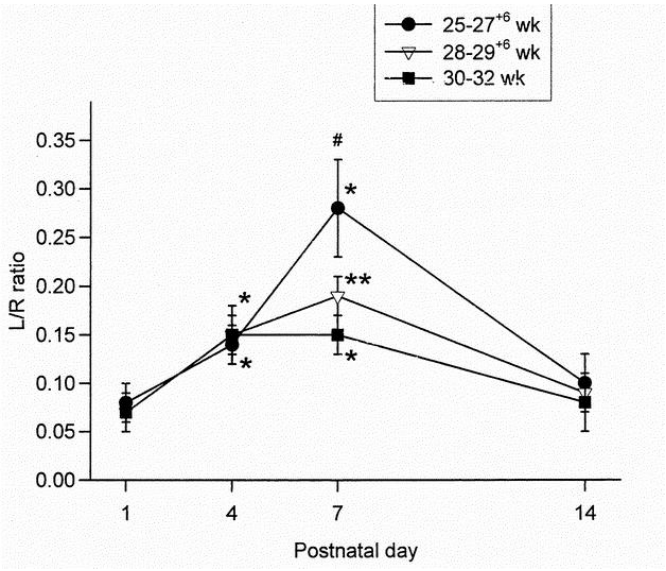


Figure 25 : Développement de la perméabilité digestive du nouveau-né.
Le ratio L/R (lactulose/l-rhamnose) a été calculé pour évaluer la perméabilité intestinale. Les auteurs ont montré que ce ratio augmentait dans les premiers jours de vie, notamment chez les enfants nés avant 27 semaines, puis diminuait au 14ème jour de vie (Rouwet et al. 2002).

iii. Maturation du contrôle de la perméabilité digestive par le SNE : exemples animaux

L'évolution post-natale du contrôle de la barrière épithéliale a été étudiée sur le côlon du porcelet (Figure 26) (Bach and Carey 1994). Des fragments de muqueuse ont été prélevés et montés en chambre de Ussing. Il a été montré une augmentation post-natale du flux ionique au travers la muqueuse (Isc, courant de court-circuit), de la différence de potentiel et de la conductance de la muqueuse. La stimulation nerveuse par EFS (electrical field stimulation) induisait une augmentation des différents paramètres, inhibée par l'usage de TTX montrant ainsi le rôle essentiel du SNE sur le contrôle de la perméabilité en période néonatale.

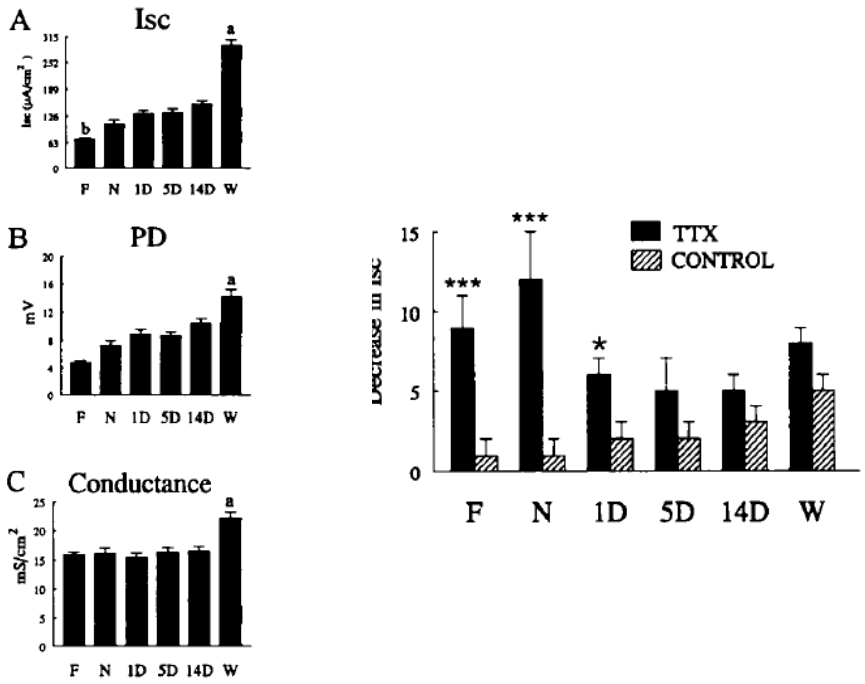


Figure 26 : Développement du transport ionique du côlon distal du porcelet.
De fragments de muqueuse colique ont été monté en chambre de Ussing en période fœtale (F), à la naissance (N), à 1, 5, 14 jours de vie (1D, 5D, 14D) et au sevrage (W). Il a été observé au cours du temps une augmentation du courant de court-circuit (Isc) (A), de la différence de potentiel transmembranaire (PD) (B) et de la conductance (C). Un traitement par tétrodotoxine (TTX) a permis d'augmenter significativement l'Isc, notamment en période fœtale, à la naissance et à 1 jour de vie (Bach and Carey 1994).

La perméabilité para-cellulaire a également été évaluée chez le porcelet (Figure 27). Le passage de dextran (4 kDa), du milieu luminal vers la circulation sanguine est maximal à 10 jours de vie puis diminue progressivement avec le temps. Sur des fragments de muqueuse colique testés *ex vivo* en chambre de Ussing, le flux de FD4 augmente de la naissance à 14 jours de vie puis diminue (De Quelen *et al.* 2011).

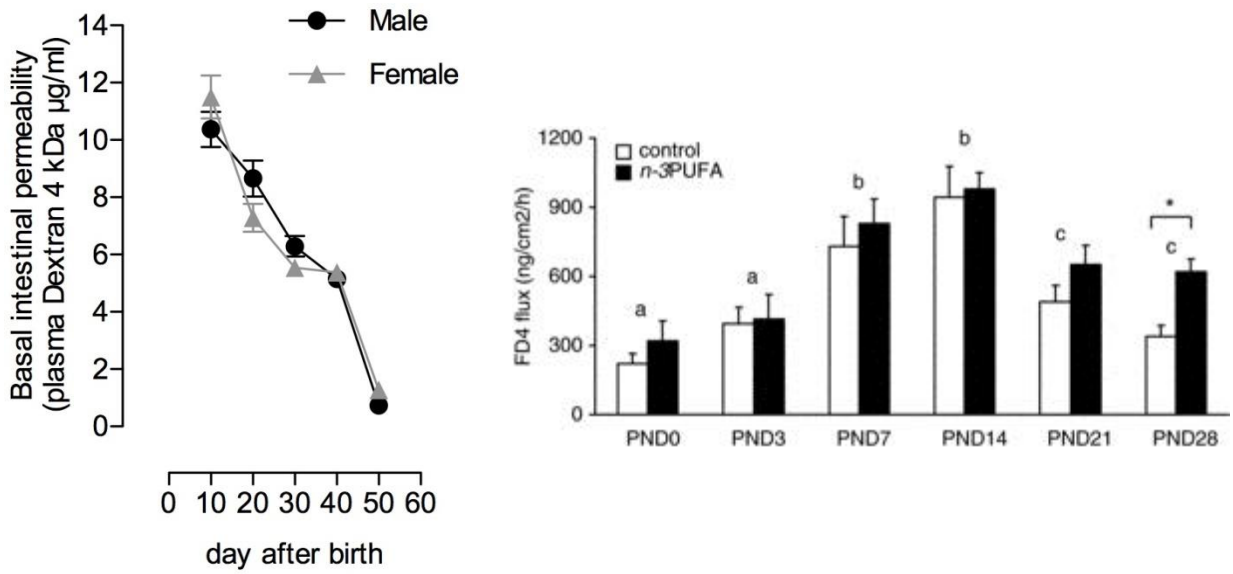


Figure 27 : Développement de la perméabilité para-cellulaire colique du porcelet.
La perméabilité para-cellulaire a été evaluée *in vivo* en mesurant le taux de Dextran plasmatique après gavage et *ex vivo* en chambre de Ussing sur des fragments de muqueuse colique. Les auteurs ont montré que la perméabilité intestinale para-cellulaire mesurée *in vivo* diminuait progressivement dès le 10ème jour de vie. *Ex vivo*, la perméabilité para-cellulaire augmentait progressivement de la naissance au 14ème jour de vie et diminuait ensuite (De Quelen et al. 2011).

4- Pathologies digestives pédiatriques caractérisées par des lésions du système nerveux entérique

Certaines pathologies digestives rencontrées en pédiatrie ont la particularité de présenter des lésions du SNE responsables d'anomalies fonctionnelles i.e. de la motricité et/ou de la perméabilité intestinale. On retrouve essentiellement des pathologies du développement comme la maladie de Hirschsprung, les pseudo-obstructions intestinales chroniques (POIC) ou des pathologies caractérisées par un défaut de maturation comme les troubles digestifs du prématuré, du nouveau-né présentant un retard de croissance intra-utérin, de l'atrésie intestinale ou du laparoschisis. Nous développerons dans un premier temps les troubles digestifs du prématuré, sujet central de notre démarche de recherche translationelle. Dans un deuxième temps nous décrirons les anomalies du SNE observées dans l'atrésie intestinale et le laparoschisis car elles évoquent des troubles du processus de maturation et ont été étudiées à la fois chez l'homme et sur modèle animal.

Les troubles des fonctions digestives du prématuré sont présents sur tous les segments du tube digestif : difficultés à la coordination succion-déglutition, retard à la vidange gastrique, reflux gastro-œsophagien, un ralentissement du transit. Il en résulte des difficultés à obtenir un apport nutritionnel entéral correspondant aux besoins de l'enfant, et en conséquence, la nécessité d'une alimentation parentérale prolongée. La tolérance alimentaire est évaluée par la survenue de vomissements, de résidus gastriques à distance du bolus alimentaire, de la présence d'un ballonnement abdominal.

Gordon a récemment classé les troubles digestifs du prématuré en 3 stades de gravité croissante en fonction des moyens à mettre en œuvre pour leur prise en charge (Gordon *et al.* 2007). Le stade 1 constitue le concept « d'intolérance alimentaire » où l'état clinique du nouveau-né ne permet pas l'apport suffisant de nutriments par voie entérale. Ce stade est théoriquement transitoire et nécessite uniquement des mesures adaptives d'alimentation afin d'attendre la maturation nécessaire à l'établissement d'une nutrition entérale complète. Toutefois, le stade 1 peut s'aggraver et aboutir à la survenue d'un stade 2 ou 3. Le stade 2 nécessite un traitement médical spécifique et les entités nosologiques

sont l'entérocolite ulcéro-nécrosante et l'entérite virale. Les stades 3 nécessitent une intervention chirurgicale comme l'entérocolite ulcéro-nécrosante ou la perforation intestinale isolée. Les facteurs associés à la survenue de ces maladies sont exposés dans une publication en annexe. De manière interssante, des anomalies majeures du SNE ont été observées dans le plexus myentérique de segments digestifs atteints d'entérocolite ulcéro-nécrosante où il a été constaté une baisse importante du nombre de neurones myentériques nitrergiques et des cellules gliales (Zhou *et al.* 2013).

L'atrésie intestinale est également une pathologie où des anomalies du SNE ont été retrouvées. Cette pathologie consiste en une interruption de la continuité digestive, associée à un defect plus ou moins important de longueur de tube digestif. L'hypothèse physiopathologique principale est la survenue d'un accident vasculaire lors de la période fœtale induisant une nécrose digestive localisée et une cicatrisation du tractus non communicante. Il en résulte une occlusion intestinale prénatale avec une dilatation digestive en amont du segment atrétique situé le plus souvent sur l'intestin grêle. Le traitement chirurgical consiste en une remise en continuité digestive en période néonatale. En fonction du contexte de naissance et/ou des constatations opératoires il est parfois nécessaire de procéder à une dérivation digestive temporaire (stomie). Les suites post-opératoires sont le plus souvent marquées par des troubles digestifs caractérisés par un transit ralenti, une mauvaise tolérance de l'alimentation entérale conduisant à des séjours prolongés en réanimation. Une des hypothèses avancée est que l'absence de stimulation luminale par les ingestats prénataux induirait un défaut de maturation des constituants du SNE. Dans ce contexte, un modèle animal d'atrésie intestinale par ligature digestive chez l'embryon de rat a permis de montrer des modifications morphologiques et fonctionnelles au sein du SNE. Il a été constaté de part et d'autre du segment atrétique que la densité des neurones Hu était diminuée, la surface neuronale augmentée, le nombre de cellules gliales inchangé et le ratio cellule gliale/neurone abaissé (Figure 28). La proportion de neurones cholinergiques (ChAT-IR) était plus élevée en amont qu'en aval alors que la proportion de neurones nitrergiques (nNOS-IR) était inchangée. D'un point de vue fonctionnel, composante cholinergique a été

explorée en étudiant la réponse contractile au carbachol des segments d'amont qui était plus intense que celle des segments d'aval (Khen-Dunlop *et al.* 2013).

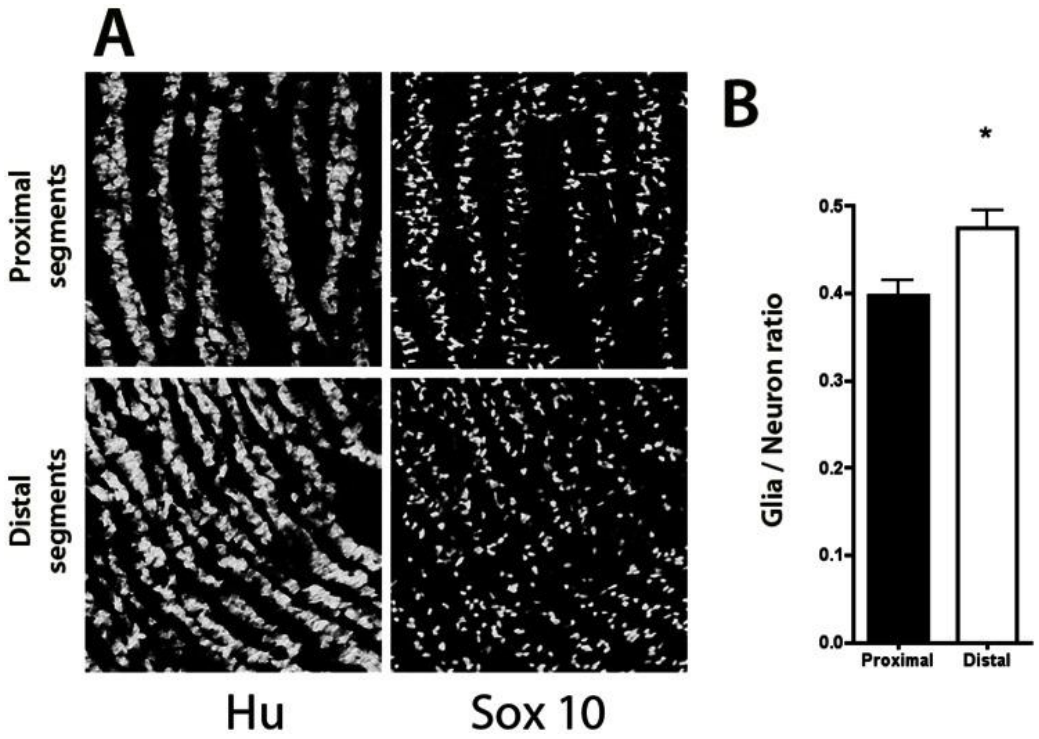


Figure 28 : Microphotographies (X40) de plexus myentériques d'intestins de rat en amont et en aval d'une atrésie intestinale expérimentale.
La densité des neurones Hu est plus importante en amont du segment atrétique alors que la densité des cellules gliales entériques SOX10 était similaire en amont et en aval de l'atrésie. Il en résulte une augmentation du ratio cellule gliale/neurone (Khen-Dunlop *et al.* 2013).

Le laparoschisis est un autre exemple de fœtopathie associée à des troubles de la motricité digestive. Il s'agit d'un defect de la paroi abdominale antérieur survenant 6 à 8 semaines après conception induisant l'issue des viscères libres dans la cavité amniotique. Il n'y a pas de membrane péritonéale ou de sac herniaire autour des anses digestives qui sont au contact direct du liquide amniotique. Le mécanisme de survenue de ce defect pariétal est probablement d'origine vasculaire (hypothèse de l'involution de la veine ombilicale droite). De manière intéressante, l'aspect macroscopique de l'intestin éviscéré à la naissance est variable, notamment l'intensité de la périviscérite (épaississement des parois digestives) qui serait la conséquence de l'exposition de l'intestin au liquide amniotique et du diamètre du collet (compression vasculaire). L'intensité des lésions observée serait un facteur prédictif majeur de la durée d'hospitalisation, de la durée de nutrition parentérale reflet de l'autonomisation digestive. La prise en charge chirurgicale consiste en une réintégration en 1 ou 2 temps des viscères extériorisés en période postnatale immédiate. Dans ce contexte, un modèle animal de laparoschisis a été mis au point par incision de la paroi abdominale antérieur du raton à E18. Ce modèle a permis de mettre en évidence les lésions de périviscérite observées chez l'homme. De manière intéressante, l'aspect morphologique du plexus myentérique de l'intestin extériorisé des ratons nouveau-nés correspondait à un stade de développement observé à E18 voire même E16 (Figure 29). Il a également été montré une baisse de l'activité acétylcholinestérasique et de l'activité de NO synthase. Concernant les cellules gliales, il a été observé une augmentation du marquage GFAP sans modification du marquage S100β. Par ailleurs, la densité des cellules interstitielles de Cajal était également fortement abaissée (Auber *et al.* 2013).

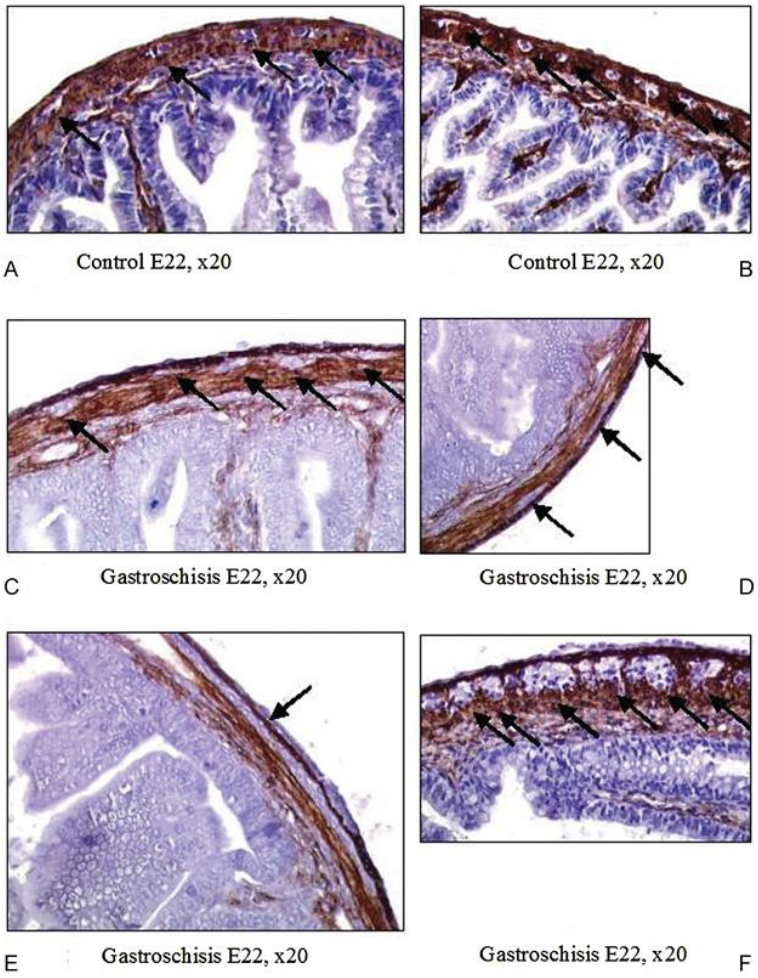


Figure 29 : Microphotographies (X20) d'intestins grêles dans un modèle de laparoschisis expérimental chez le rat avec marquage anti α-smooth-muscle actin.
Les photographies A et B illustrent l'aspect retrouvé chez les animaux contrôles avec un plexus myentérique (flèches noires) d'aspect mature. Un aspect immature (C, D) ou très immature (E, F) a été retrouvé chez les animaux présentant un laparoschisis (gastroschisis) (Auber *et al.* 2013).

B- Rôle du microbiote sur la maturation du système nerveux entérique

La place de la flore intestinale ou microbiote dans le fonctionnement des grandes fonctions physiologiques du vivant est de plus en plus étudiée. Dans de nombreuses situations pathologiques des modifications de la flore ont été mises en évidence, comme les maladies inflammatoires de l'intestin mais aussi dans des pathologies extradigestives comme l'obésité, l'asthme, l'autisme d'autres pathologies psychiatriques.

L'étude des interactions entre l'hôte et le microbiote est extrêmement complexe et fait l'objet de nombreuses publications. L'approche thérapeutique par manipulation de la flore intestinale est une voie de recherche innovante et présente un enjeu commercial important.

Cette partie est organisée en 6 chapitres. Tout d'abord nous allons exposer des données illustrant le lien entre le microbiote et les composants du SNE. Nous décrirons ensuite les moyens d'étude du microbiote permettant l'étude de sa composition et de sa mise en place. Une brève description du rôle de la flore dans l'homéostasie du tube digestif sera suivie de la mise en évidence de l'impact du microbiote sur les fonctions digestives.

1- Le système nerveux entérique est une cible pour le microbiote

L'apport de bactéries sur des animaux vivants a permis de modifier le phénotype de neurones entériques. Des porcelets ont été supplémentés par une levure, *Saccharomyces boulardii*, et il a été montré une baisse de la proportion de neurones calbindin immunoréactifs (Kamm *et al.* 2004). D'autres porcelets ont été supplémentés par *Pediococcus acidilactici* et ont présenté une augmentation du nombre de neurones CGRP+ et galanin+ dans les ganglions du plexus sous muqueux de l'iléon (di Giancamillo *et al.* 2010).

Une autre étude portant sur l'augmentation de la sensibilité viscérale induite par utilisation d'antibiotiques a montré que *Lactobacillus paracasei* pouvait atténuer l'hyperalgésie constatée (Verdú *et al.* 2006). De même, *Lactobacillus acidophilus* a induit une augmentation d'expression des recepteurs opioïdes et cannabinoïdes dans un modèle d'hypersensibilité viscérale colique (Rousseaux *et al.* 2007).

Une des explications possible dans les effets des bactéries sur les composants du SNE impliquerait les PRRs (pattern- recognition receptors) et en particulier les Toll-like receptors (Rumio *et al.* 2006; Barajon *et al.* 2009; Takeuchi and Akira 2010). Il s'agit d'une famille de protéines capables de reconnaitre des composants de micro-organismes composant la flore. Ces molécules semblent jouer un rôle majeur dans l'immunité innée et dans la reconnaissance des micro-organismes pathogènes ou commensaux (Prescott, Lee and Philpott 2013). Les Toll-like receptors (TLR) sont des protéines transmembrannaires comportant un domaine extracellulaire de reconnaissance, un domaine transmembranaire et un domaine intracellulaire permettant la transduction du signal d'activation. Il existe 13 types de TLR chez l'homme. Il a été montré que les TLR3 et 7 (reconnaissant l'ARN viral ou parasitaire), TLR 2 (reconnaissant les peptidoglycanes des bactéries à gram positif) et le TLR 4 reconnaissant le LPS (lipo-polysaccharide, composant des bactéries à gram négatif) sont exprimées par les neurones et la glie entérique (Figure 30) (Rumio *et al.* 2006; Barajon *et al.* 2009; Takeuchi and Akira 2010). Ainsi, *in vitro*, sur des plexus myentériques isolés de rats, le LPS agit directement sur les CGE induisant une augmentation de production de cytokines pro inflammatoires Il 1 et PGE2 (Murakami, Ohta and Ito 2009). Les souris TLR4-/- possèdent

moins de neurones nNOS positifs, à l'image des animaux axéniques et des souris traitées par antibiotiques (Anitha *et al.* 2012). Les souris TLR2-/- présentent également moins de neurones myentériques, notamment les neurones nNOS positifs. D'un point de vue fonctionnel, ces souris présentent des anomalies de motricité digestive et une modification de la sécrétion de chlore sur des explants iléaux. De même, il a été constaté une baisse d'expression des marqueurs gliaux GFAP and S100β. De façon intéressante, l'addition de GDNF réduisait ces déficits (Brun *et al.* 2013).

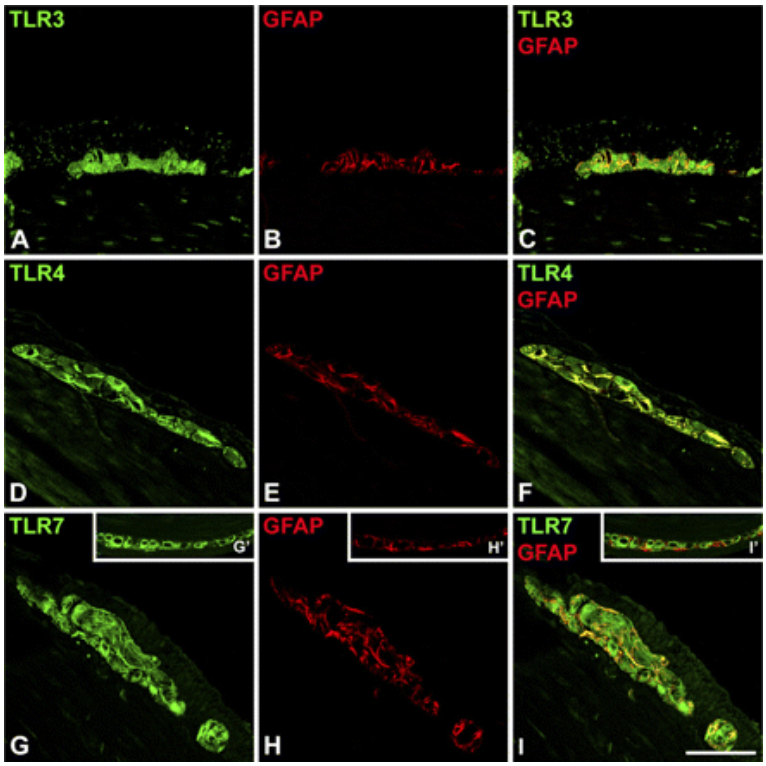


Figure 30 : Expression des Toll Like Receptors par les cellules gliales entériques. Microphotographies confocales de plexus myentérique murin avec immuno-marquage de toll like receptors (TLR) : TLR 3 (A), TLR 4 (D), TLR 7 (G) et GFAP (B, E, H). Sur les images superposées (C,F,I) on constate une co-localisation des marquages gliaux et TLR. Echelle 30 µM (Barajon *et al.* 2009).

2- Moyens d'étude du microbiote intestinal

L'analyse des publications scientifiques concernant l'étude du microbiote nécessite une bonne compréhension des moyens d'étude de celui-ci qui se heurtent à de nombreux biais techniques Les outils d'exploration sont distingués en procédés culture-dépendants et procédés culture-indépendants. Ces deux approches sont complémentaires avec leurs propres avantages et inconvénients. De plus, la flore présente une forte variabilité inter-individuelle et intra-individuelle en fonction du prélèvement analysé : le type de prélèvement (contenu digestif versus paroi digestive) et le lieu de prélèvement (fèces émise versus liquide digestif prélevé par exemple).

TAXONOMIE

La taxonomie est une discipline qui a pour but de regrouper les organismes vivants en taxons. Le système de classification de Cavalier-Smith (Cavalier-Smith 1998) distingue deux empires (procaryotes et eucaryotes) répartis en 6 règnes : les bactéries pour l'empire des procaryotes ; et les eucaryotes composés des protozoaires, les chromistes, les plantes, les animaux et les champignons. Puis la classification hiérarchique du monde des vivants s'organise par :

- Embranchement, ou division, ou phylum
    - Classe, ± sous classe
        - Ordre
            - Famille
                - Genre
                    - Espèce
                        - Forme

TECHNIQUES D'EXPLORATION DE LA COMPOSITION DU MICROBIOTE CULTURE DEPENDANT

Ces techniques historiques reposent sur des procédés de cultures sélectives basées sur la morphologie microbienne, les spécificités biochimiques ou physiologiques des micro-organismes étudiés. L'avantage de cette approche est la possibilité d'étudier des agents vivants et d'en approcher leurs fonctions physiologiques. Ces techniques permettent également d'isoler des bactéries, même à de faibles densités ($<10^5$ UFC, unités formant des colonies), de les purifier et d'engendrer des cultures pures. L'exploration de la flore intestinale est limitée par le fait que 40 à 90% des bactéries composant le microbiote ne sont pas cultivables par les techniques usuelles de laboratoire (Zoetendal *et al.* 2004b; Bik *et al.* 2010). Ainsi, seulement 20% des bactéries retrouvées par approche métagénomique sont retrouvées par cultures (Venter *et al.* 2004; Eckburg *et al.* 2005).

L'approche par « culturomique » tend à optimiser les méthodes de culture utilisant de nombreuses conditions de cultures simultanément : degré d'aérobiose, substrat, présence d'antibiotiques, l'utilisation de chocs thermiques, filtration passive, utilisation de chambres de diffusion. Les bactéries isolées sont ensuite identifiées par séquençage de l'ARN 16s ou par MALDI TOF MS (matrix-assisted laser desorption ionization–time of flight mass spectrometry).

TECHNIQUES CULTURE INDEPENDANT

Ces techniques sont basées sur l'étude du génome bactérien. Ces approches permettent une analyse plus fine de la diversité du microbiote, de manière qualitative et quantitative (Mackie, Sghir and Gaskins 1999; Zoetendal *et al.* 2004a; Eckburg *et al.* 2005). Les procédés utilisées sont basées sur la PCR, la qPCR, la FISH, la cytomètrie de flux, le séquençage ADN, les puces à ADN.

L'approche moléculaire pour étudier la diversité bactérienne utilise le plus souvent l'ARN 16s ribosomial car présent dans toutes les bactéries. Il est de petite taille (environ 1.5 kb), et possède une variabilité suffisante pour discriminer les souches et les espèces (Sekirov *et al.* 2010). L'ARN 16s est l'ARN ribosomique constituant la petite unité du ribosome des procaryotes dont la fonction est la traduction protéique de l'ARN messager. Ainsi, plus de 3

millions de séquences d'ARN 16s sont recensées sur le site du « ribosomal data project ». Une alternative est l'étude du gène cpn60 qui code une sous-unité d'une protéine chaperonne heat shock protein (HSP60/GroEL) qui permet une distinction plus précise et plus discriminante des bactéries (Hill *et al.* 2004; Dumonceaux *et al.* 2006).

Les techniques d'études de la flore culture-indépendant à haut débit (ère des « omics ») engendrent une somme de données importantes nécessitant une analyse « *in silico* ». La métagénomique étudie la composition et la fonction de la flore par séquençage de l'ensemble du génome présent dans un échantillon (Gill *et al.* 2006) et tente de prédire le métaprotéome. Cependant, de nombreuses discordances ont été montrées par métaprotéomique (Verberkmoes *et al.* 2009). La métaprotéomique consiste à analyser l'ensemble des protéines présentes dans un échantillon. Les protéines sont séparées par électrophorèse sur gel de polyacrylamide puis prélevées, et analysées par spectrométrie de masse après digestion enzymatique.

La métatranscriptomique analyse à haut débit les séquences ARN présentes dans la flore. Elle permet d'obtenir des données fonctionnelles, et aussi des données concernant la régulation d'expression d'ADN en fonction des conditions locales (alimentation, …).

La métabolomique étudie les profils des métabolites présents dans un échantillon en utilisant la spectrométrie de masse, la résonnance magnétique nucléaire (Turnbaugh and Gordon 2008) en se centrant sur les métabolites d'intérêt (acides gras à chaîne courte, acides organiques, acides aminés, uracil, lipides…)(Yap *et al.* 2008).

APPROCHE ANIMALE

L'approche animale a été utilisée pour caractériser l'impact de la flore sur le fonctionnement de l'organisme en utilisant des modèles gnotobioques, ou des modèles transgéniques. Cela permet une étude dynamique entre l'hôte, l'alimentation et le microbiote.

La gnotobiologie étudie la colonisation d'animaux axéniques avec des souches bactériennes sélectionnées issues de la flore humaine (Sekirov *et al.* 2010). Elle permet d'obtenir un modèle simplifié d'interaction. Néanmoins, la flore étant essentielle au

développement précoce de l'organisme, les animaux axéniques présentent des modifications de leur fonctionnement physiologique (cf plus bas). Par ailleurs, cette étude globale ne permet pas d'identifier d'interaction au sein de la flore elle-même.

L'utilisation d'animaux transgéniques permet d'identifier les gènes de l'hôte qui ont une interaction avec la flore. Par exemple, les souris KO Il 10 ont moins d'espèces de lactobacilles dans leur côlon, et plus de bactéries adhérentes à la muqueuse (Madsen *et al.* 1999). Les souris KO TLR 5 ont moins de *Bacteroidetes* et de *Lachnospiraceae* et présentent un syndrome métabolique (Vijay-Kumar *et al.* 2010). L'expression de la defensine humaine chez la souris a induit une modification de la composition de la flore de la souris (Salzman *et al.* 2010).

3- Le microbiote intestinal adulte

La flore intestinale, ou microbiote intestinal représente l'ensemble des micro-organismes présents dans le tube digestif soit 100.000 milliards d'entités pour un poids de 1.5 kg environ. Le microbiote se compose d'archées, de bactéries, de protistes, de levures et de virus qui constituent avec l'organisme (hôte) un exemple de mutualisme apportant des bénéfices réciproques.

Le microbiote intestinal se compose essentiellement de bactéries, les organismes eucaryotes représentant 0.5%, les archées 0.8% et les virus 5.8%. Plus de 35.000 espèces bactériennes ont été recensées dans le microbiome humain représentant près de 10 millions de gènes différents selon les travaux du projet Metagenome of the Human Intestinal tract (MetaHIT).

Parmi les plus de 70 phyla présents dans la flore intestinale, 2 dominent largement la communauté bactérienne du microbiote humain : les Firmicutes et les Bacteroidetes (Figure 31) (Arumugam et al. 2011). Les Firmicutes, phyla le plus représenté, contient plus de 200 genres bactériens différents (*Ruminococcus, Clostridium, Lactobacillus Eubacterium, Faecalibacterium* et *Roseburia*). Le phylum des bacteroidetes contient les groupes des *Bacteroides*, les *Prevotella* et *Xylanibacter*. D'autre phyla sont présents comme les Proteobacteria (*Escherichia*, *Enterobacteriaceae, Desulfovibrio*), les Actinobacteria (*Bifidobacteria*, *Collinsella*), les *Fusobacteria*, *Spirochaetes*, et *Verrucomicrobia* (Gerritsen *et al.* 2011).

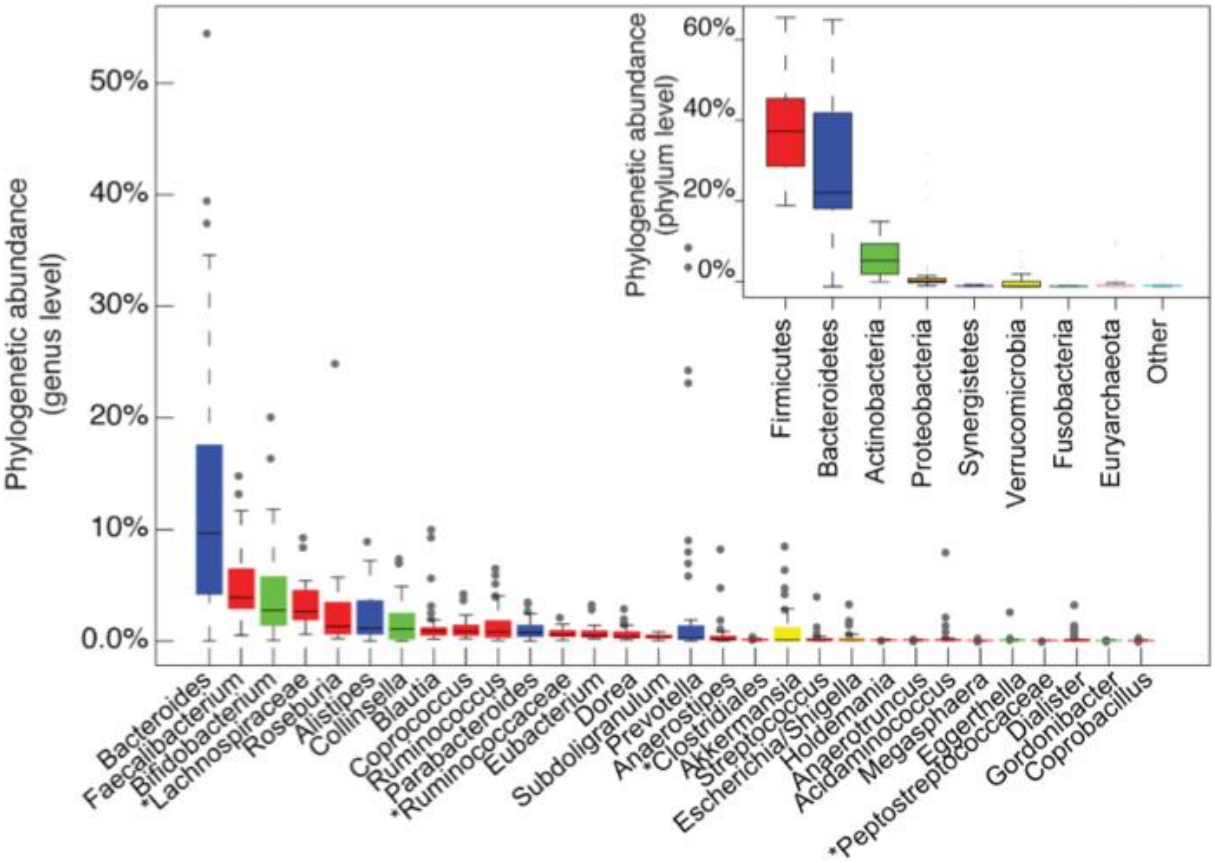


Figure 31 : Composition du microbiote intestinal adulte.
Représentation des 30 genres bactériens les plus abondants du microbiote. Les couleurs correspondent au phylum d'appartenance des genres (Arumugam et al. 2011).

Une publication danoise a étudié le microbiome de 123 individus non obèses et 169 obèses et a permis d'introduire le concept de high gene count (HGC) et low gene count (LGC), ayant des implications métaboliques (Le Chatelier *et al.* 2013). Le microbiome HGC inclus des *Anaerotruncus colihominis, Butyrivibrio crossotus, Akkermansia* sp., *et Fecalibacterium* sp. avec un ratio élevé de *Akkermansia* (Verrucomicrobia) : *Ruminococcus torque/gnavus*. D'un point de vue métabolique, ce microbiote est propice à la production de butyrate, d'hydrogène et une baisse de la production de sulfite d'hydrogène. Les individus porteurs de ce type de flore présentent moins de troubles métaboliques et d'obésité que dans le groupe LGC. Le microbiote du groupe LGC contient plus de *Bacteroides*, de *Ruminococcus gnavus* connues pour être impliquées dans les maladies inflammatoires de l'intestin.

La répartition des espèces bactérienne le long du tube digestif varie en fonction de la localisation (Figure 32). Ainsi, certaines bactéries vont cheminer le long du tractus de la bouche vers l'anus (flore de passage) alors que d'autres vont coloniser un organe particulier. Par ailleurs, le nombre de bactéries par gramme de contenu varie de 10 dans l'œsophage à plus de à 10^{12} dans le côlon. Les *Streptococcus* sont le genre dominant dans l'œsophage, le duodénum, le jéjunum, *Helicobacter* ou l'association *Streptococcus, Prevotella, Veillonella* et *Rothia* dans l'estomac. Le côlon contient plus de 70% des micro-organismes de la flore et est le plus étudié par l'analyse métagénomique des selles. En plus de la flore dominante (Firmicutes et Bacteroidetes), le microbiote colique contient des espèces pathogènes en très faible proportion (<0.1%) comme *Campylobacter jejuni, Salmonella enterica, Vibrio cholera, Escherichia coli* et *Bacteroides fragilis*.

Une autre source de variabilité est la localisation au sein de la lumière digestive. Les *Bacteroides, Bifidobacterium, Streptococcus, Enterobacteriacae, Enterococcus, Clostridium, Lactobacillus et Ruminococcus* sont retrouvées plus fréquemment dans la lumière digestive et dans les selles alors que *Clostridium, Lactobacillus, Enterococcus* et *Akkermansia* sont prédominantes au contact de la muqueuse et du mucus (Swidsinski *et al.* 2005; de Cárcer *et al.* 2011).

Le concept d'entérotype proposé par le consortium MetaHit (Arumugam *et al.* 2011) propose une classification en groupe d'associations bactériennes symbiotiques équilibrées qui présentent des fonctions métaboliques différentes. L'entérotype 1 présente une abondance de *Bacteroides* et possède une activité saccharolytique permettant la dégradation de carbohydrates. L'entérotype 2 est dominé par *Prevotella* et possède une activité mucolytique tout comme le type 3, abondant en *Ruminococcus*.

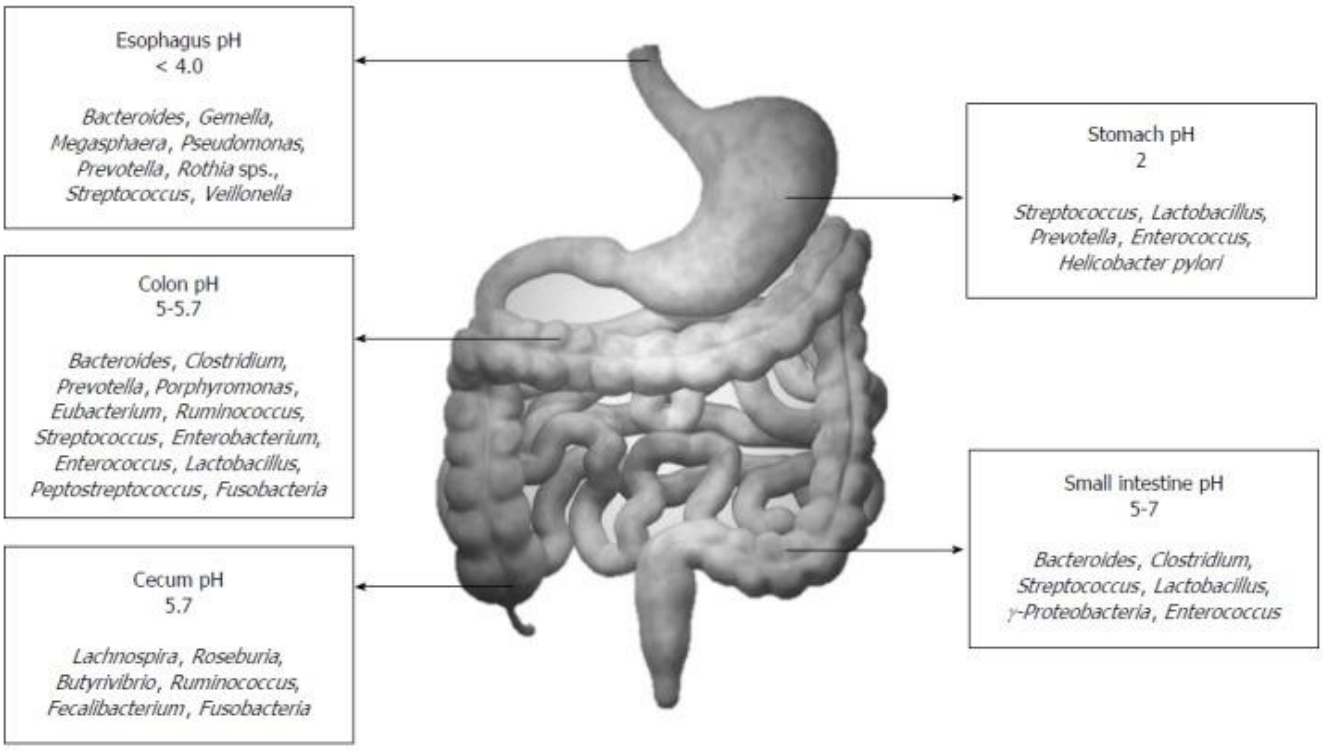


Figure 32 : Distribution du microbiote en fonction de la localisation au sein du tube digestif.
La composition du microbiote est variable en fonction de l'organe étudié (Jandhyala et al. 2015).

4- Mise en place du microbiote intestinal

a. En conditions physiologiques

L'étude de la mise en place de la flore chez le nouveau-né est historique : en 1874 Bilroth a constaté sur des observations microscopiques que le méconium ne contenait pas de bactéries alors que les premières selles (après issue complète du méconium) en contenaient. Ces observations ont été confirmées par Escherich en 1885 par culture.

La composition de la flore possède de nombreuses sources de variabilité inter et intra-individuelles. Il a été montré que la variabilité intra-individuelle était majoritairement expliquée par l'âge (Lozupone *et al.* 2013). La complexité de la composition du microbiote s'accroit avec le temps (Figure 33).

L'analyse de larges cohortes américaines, africaines et européennes a montré qu'avant 3 ans la composition de la flore intestinale était instable, soumise aux changements environnementaux (De Filippo *et al.* 2010; Koenig *et al.* 2011; Yatsunenko *et al.* 2012). De plus, la flore de l'enfant présente également une plus faible diversité, environ 1000 unités taxonomiques opérationnelles (operational taxonomic units ou OTUs) contre plus de 2000 chez l'adulte et une plus grande variabilité inter-individuelle (Yatsunenko *et al.* 2012).

Le tube digestif du nouveau-né est un environnement aérobie où seules les bactéries anaérobies facultatives peuvent croitre, comme les *Enterobacteriaceaes*. Quelques jours plus tard, le milieu devient anaérobie permettant la colonisation de souches anaérobies strictes telles que *Bifidobacterium*, *Clostridium*, et *Bacteroides* (Matamoros *et al.* 2013).

Dans les premières semaines de vie, la composition de la flore intestinale est à l'image de la flore maternelle cutanée et vaginale contenant majoritairement *Enterococcaceae*, *Streptococcaceae*, *Lactobacillaceae*, *Clostridiaceae*, et *Bifidobacteries*. L'alimentation lactée permet ensuite le développement de bactéries fermentant les oligosaccharides comme les *Bifidobacterium* considérées comme les bactéries prédominantes à ce stade (Lahtinen *et al.* 2009; Aires *et al.* 2011; Turroni *et al.* 2012; Lozupone *et al.* 2013).

La diversification alimentaire est une étape importante en apportant une variété de nutriments, polysaccharides, fibres non digestibles par les enzymes produites par l'organisme. Cette période voit l'apparition des *Bacteroides*, *Clostridium*, *Ruminococcus* associée à une baisse de la proportion des *Bifidobacterium* et des *Enterobacteriaceae* (Fallani *et al.* 2011; Koenig *et al.* 2011). Puis la composition de la flore s'apparente de plus en plus à la flore de type adulte contenant principalement des *Ruminococcaceae, Lachnospiraceae, Bacteroidaceae,* et *Prevotellaceae* (Lozupone et al. 2013).

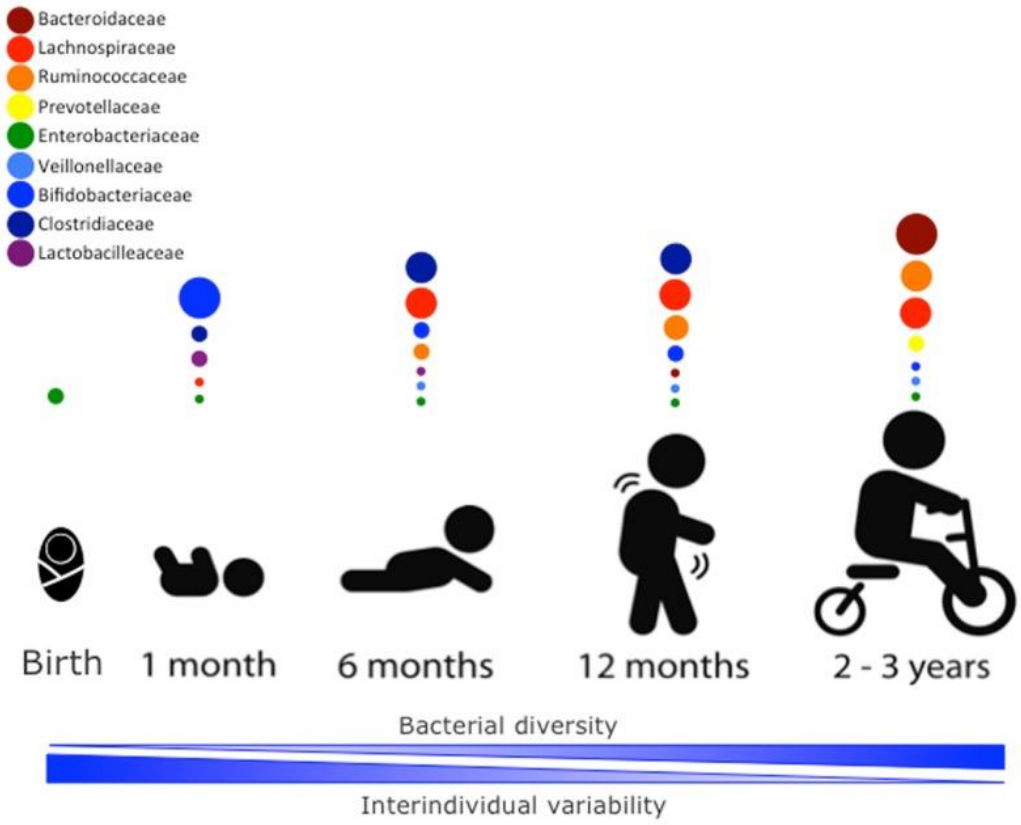


Figure 33 : Etapes de la colonisation microbienne chez l'enfant.
A la naissance le microbiote se compose essentiellement d'*enterobacteria*, puis dans les premiers jours de vie la flore est dominée par des bactéries anaérobies strictes. Les bifidobactéries apparaissent dans les premiers mois de vie, puis la diversification alimentaire s'accompagne de l'apparition d'espèces de *Clostridium* (*Lachnospiracea*, *Clostridiaceae*, *Ruminococcaceae*). Vers 2-3 ans les *Bacteroideceae* deviennent dominantes (Arrieta et al. 2014).

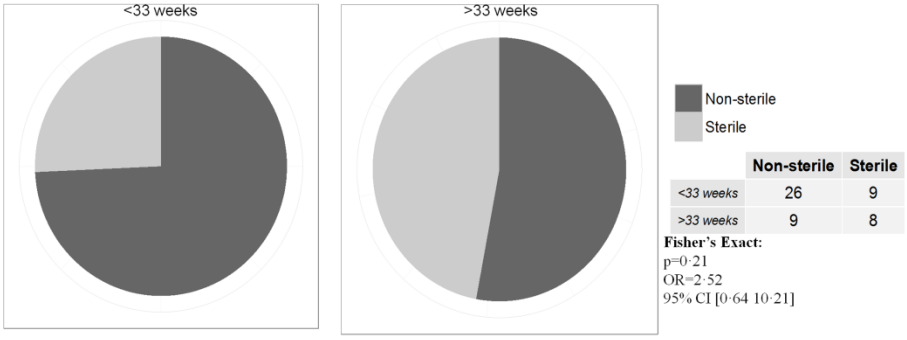
b. Facteurs modifiant la mise en place de la flore

La colonisation du tube digestif par la flore est un phénomène dynamique qui est soumis à de nombreux perturbateurs. Il en résulte une composition unique, personnelle, qui introduit le concept d'empreinte. Les jumeaux homozygotes présentent une composition de flore proche avec néanmoins quelques variabilités.

i. Période prénatale

La colonisation du tube digestif par la flore intestinale intervient essentiellement à la naissance. Néanmoins, des études récentes ont montré que le fœtus était exposé à certaines bactéries *in utero* par le biais du liquide amniotique (DiGiulio *et al.* 2008; DiGiulio 2012). Il a été retrouvé des bactéries comme *Mycoplasma* et *Ureaplasma* dans des situations pathologiques comme l'accouchement prématuré et l'entérocolite ulcéro-nécrosante (Goldenberg and Culhane 2003; Okogbule-Wonodi *et al.* 2011; Kwak *et al.* 2014). De même, les mères présentant une infection vaginale ont un risque d'accouchement prématuré augmenté (Menon *et al.* 2011). D'un autre côté, des germes ont été retrouvés dans des prélèvements de liquides amniotiques ou de placentas sans aucun impact clinique chez la mère ou l'enfant (Steel *et al.* 2005; Satokari *et al.* 2009; Aagaard *et al.* 2014).

Il a été également montré récemment que le méconium, n'était pas toujours stérile (Figure 34) (Mshvildadze *et al.* 2010; Gosalbes *et al.* 2013; Hu *et al.* 2013) ce qui supporte l'hypothèse que les bactéries du liquide amniotique ont accès au tube digestif du fœtus. Une autre étude a montré également que la flore retrouvée dans le méconium du prématuré était plus proche de celle retrouvée dans le liquide amniotique plutôt qu'à celle de la bouche ou du vagin de la mère (Ardissone *et al.* 2014).



	Non-sterile	Sterile
<33 weeks	26	9
>33 weeks	9	8

Fisher's Exact:
p=0·21
OR=2·52
95% CI [0·64 10·21]

Figure 34 : Colonisation microbienne du méconium.
Le microbiote du méconium a été analysé chez des nouveau-nés de moins de 48h de vie. Le méconium des enfants nés avant 33 semaines était plus souvent colonisé que celui des enfants nés après 33 semaines (Ardissone et al. 2014).

Ainsi ont été retrouvées chez le nouveau-né sain des *Enterobacteriaceae* (dont *Escherichia coli* et *Shigella spp*.), *Enterococci, Streptococci, Staphylococci* (dont *Staphylococcus epidermidis*), et *Bifidobacteria* (Jiménez et al. 2008; Gosalbes et al. 2013). Chez la rate gestante, *Enterococcus faeciuma* administré oralement pendant la gestation a été retrouvée dans le méconium de ratons nés par césarienne et sacrifiés juste après la naissance (Jiménez et al. 2008).

Le mode d'accouchement est également un facteur important dans la composition de la flore. Les enfants nés par césarienne présentent plus fréquemment des *Staphylococcus, Corynebacterium, Propionibacterium* par rapport aux enfants nés par voie basse qui eux présentent plus de *Lactobacillus,* de *Prevotella* (Dominguez-Bello et al. 2010; Matamoros et al. 2013). Ce facteur est important car, à la différence de l'exposition faible lors de la grossesse, le mode d'accouchement conditionne le premier contact avec un inoculum massif : soit par la flore cutanée soit par la flore vaginale (Biasucci et al. 2008; Dominguez-Bello et al. 2010).

Ainsi, à 3 jours de vie, les enfants nés par césarienne présentent moins d'espèces de *Bifidobacterium* ou de *Bacteroides* que les enfants nés par voie basse (Adlerberth et al. 2006; Penders et al. 2006; Biasucci et al. 2008). A 1 an, l'absence de *Bacteroidetes* et une plus faible diversité bactérienne à 2 ans montrent que l'effet du mode d'accouchement se prolonge dans le temps (Jakobsson et al. 2014).

Le terme d'accouchement est également un facteur important dans la mise en place de la flore. Il a été montré qu'un accouchement prématuré était associé à une présence plus importante de *Clostridium difficile* et de *Staphylococcus* (Penders et al. 2006). Néanmoins chez le prématuré il semble que le mode d'accouchement ne soit pas un facteur majeur de variabilité dans la composition de la flore (Arboleya et al. 2012) qui sont dominés par l'utilisation d'antibiotiques, et l'environnement hospitalier de réanimation (Berrington et al. 2013).

Dans ce contexte, des études épidémiologiques ont montrées que la césarienne pourrait être un facteur de risque de développer à long terme des maladies inflammatoires chroniques de l'intestin chez l'enfant, en dehors de toute histoire familiale (Bager et al. 2012), de maladie cæliaque (Decker, Hornef and Stockinger 2011) ou d'obésité (Blustein et al. 2013).

ii. Allaitement maternel

Le lait maternel est réputé comme l'aliment le plus adapté au nourrisson. Il possède lui aussi son propre microbiote. Le lait maternel contient également des oligosaccharides qui permettent la croissance de certaines souches bactériennes (Fernández *et al.* 2013). Ainsi un nourrisson absorbe 10^5 à 10^7 bactéries par jour (Heikkilä and Saris 2003) comme les *Bacteroides* et *Clostridia* (Jost *et al.* 2013) qui composent la flore intestinale et sont retrouvés dans les selles des nourrissons allaités (Martín et al. 2012). La flore de ces nourrissons allaités par leur mère contient plus de *Bifidobacteria* et de *Lactobacillus* alors que la flore des nourrissons en allaitement artificiel contient plus de *Bacteroides*, *Clostridium*, *Streptococcus*, *Enterobacteria*, et de *Veillonella* spp (Adlerberth and Wold 2009; Fallani *et al.* 2010; Bezirtzoglou, Tsiotsias and Welling 2011; Sahl, Matalka and Rasko 2012). La présence d'oligosaccharides dans le lait maternel pourrait être un facteur explicatif permettant la croissance des bifidobactéries qui ont la capacité de les métaboliser (Sela and Mills 2010). En conséquence, du lactate et des acides gras à chaîne courte sont produits, responsables de l'acidification du milieu ayant un rôle protecteur vis-à-vis de certains germes pathogènes (Yu *et al.* 2013).

iii. Le sevrage

Une des fonctions majeure de la flore réside dans l'optimisation de la digestion. En effet, certaines bactéries présentes dans le tube digestif possèdent la machinerie enzymatique capable de dégrader les aliments non digérés par les secrétions digestives (Flint *et al.* 2012). Ainsi, la flore est responsable de la dégradation de fibres alimentaires, des polysaccharides, de la cellulose, du xylane (Tremaroli and Bäckhed 2012). Il en résulte une production d'acides gras à chaîne courte source d'énergie pour les cellules épithéliales (Smith *et al.* 2013).

Le type de carbohydrates consommé conditionne les proportions d'acides gras à chaînes courte et des bactéries qui les produisent (Tremaroli and Bäckhed 2012; Smith *et al.* 2013; Trompette *et al.* 2014). En comparant la composition de la flore avant et après sevrage, il a été montré une baisse significative des proportions de *Bifidobacteria,* d'*Enterobacteria,* de *Clostridium difficile* et de *Clostridium perfringens* et une augmentation de proportion de *Clostridium coccoides* et *Clostridium leptum* (Fallani *et al.* 2011).

D'autres facteurs semblent également modifier la composition de la flore comme la supplémentation en fer : baisse de *Bifidobacteria rothia* et *Lactobacillaes* (Krebs *et al.* 2013), la consommation de viande : augmentation de *Clostridium* du groupe XIVa (productrices de butyrate), la consommation de pois : augmentation des *Bacteroidetes* (Koenig *et al.* 2011).

iv. Utilisation d'antibiotiques

Après sa mise en place, la flore reste stable au cours du temps. Lors de l'utilisation d'antibiotiques sur de courtes périodes la flore retrouve sa composition habituelle introduisant le concept de résilience (Lozupone *et al.* 2012). Par contre, l'utilisation prolongée d'antibiotiques chez l'enfant semble induire des modifications à long terme (Fouhy *et al.* 2012) se manifestant par une baisse de sa diversité, et la possibilité de développement de pathogènes comme *Clostridium difficile* (Doorduyn *et al.* 2006; Dethlefsen *et al.* 2008; Rousseau *et al.* 2011).

Chez le nouveau-né, l'utilisation précoce d'ampicilline et de gentamycine a induit une augmentation des proportions de *Proteobacteria*, *Actinobacteria*, et de *Lactobacillus* comparé aux enfants non traités, et ce jusqu'à 1 mois après l'arrêt du traitement (Fouhy *et al.* 2012). L'utilisation d'antibiotiques dans les 4 premiers jours de vie induit également une baisse de la diversité microbienne avec une baisse des *Bifibobacterium* et une augmentation des *Enterococcus* (Tanaka *et al.* 2009).

Par ailleurs, un déséquilibre de la composition de la flore, appelé dysbiose, a été retrouvé dans de nombreuses pathologies digestives (intestin irritable, maladies inflammatoires de l'intestin) et extra-digestives (asthme, diabète de type 1) (Russell *et al.* 2012). Un des facteurs de risque de survenue de dysbiose à l'âge adulte pourrait être des perturbations lors de la mise en place de la flore. En effet, l'utilisation précoce en période néonatale d'antibiotiques serait un facteur associé à l'asthme allergique ou plus largement à l'atopie (Penders *et al.* 2007; Russell *et al.* 2012).

5- Microbiote et homéostasie digestive

a. Rôle métabolique de la flore intestinale

Les micro-organismes de la flore tirent leur énergie de la dégradation des carbohydrates d'origine digestive, notamment par fermentation des fibres par *Bacteroides, Roseburia, Bifidobacterium, Fecalibacterium,* et *Enterobacteria*. Il en résulte la production d'acides gras à chaîne courte comme le butyrate, le propionate, l'acétate qui sont également source d'énergie pour les cellules épithéliales (Jandhyala *et al.* 2015). Une des applications potentielle serait l'apport de substrat bactérien afin d'augmenter la production de ces acides gras. Chez le raton, l'apport de galacto-oligosaccharides a permis ainsi une augmentation significative de butyrate dans le côlon (Barrat *et al.* 2008), tout comme chez le rat adulte avec un régime riche en fibre (Soret *et al.* 2010).

Certains micro-organismes permettent de limiter l'accumulation de toxiques comme le D-lactate (*Bacteroides thetaiomicron*), l'oxalate (*Oxalobacter formigenes,* certains *Lactobacillus,* et *Bifidobacterium*) et la dégradation des sels biliaires (*Bacteroides intestinalis*, *Bacteroides fragilis* et *Escherichia coli*) (Jandhyala *et al.* 2015).

Par ailleurs, la composition de la flore aurait un rôle sur le métabolisme des lipides, permettant de moduler l'activité de lipoproteines lipases, ou en augmentant la production de la colipase nécessaire à l'action de la lipase pancréatique. L'activité protéasique et peptidasique de la flore permet également la production de peptides anti-microbiens (bactériocines), de GABA. Enfin, la flore permet également la production de vitamines K et B.

b. Rôle de la flore sur l'immunité

L'homéostasie du tube digestif permet un équilibre entre la tolérance de bactéries commensales bénéfiques pour l'hôte et la prévention de la croissance de germes résidents pathogènes. Un des mécanismes impliqué est la présence d'une double couche de mucus qui permet d'isoler la muqueuse des bactéries de la lumière digestive, notamment au niveau du côlon. Le mucus est un film de glycoprotéines, de mucines d'une épaisseur de 150 µm. ce film est stabilisé par le trefoil-factor et les resistin-like molecules β secrétés par les cellules de Goblet. La couche interne de mucus, au contact des cellules épithéliales, est dense, dépourvue de micro-organismes, alors que la couche externe constituée de glycanes contient des bactéries (Johansson, Larsson and Hansson 2011).

L'épaisseur de la couche de mucus est plus faible dans l'intestin grêle ce qui nécessite d'autres mécanismes antimicrobiens, produits par les cellules de Paneth (cathelicidins, C-type lectins, pro-defensins) par activation des pattern recognition receptor (PRR) dont les Toll like receptors (TLRs), C-type lectin receptors (CLRs) et oligomerisation domains (NOD) like receptors (NLRs) (Takeuchi and Akira 2010).

Les PPRs sont activés par des specific microbe-associate molecular patterns (MAMPs), notamment les peptidoglycanes, lipopolysaccharides (LPS), lipide A, flagelles et ARN/ADN bactériens.

Il a été ainsi montré que *Bacteroides thetaiotaomicron* pouvait induire une production de metalloproteinase matrilysin clivant les prodéfensines en défensines actives (López-Boado *et al.* 2000). De même, *Lactobacillus* sp. induit une production d'acide lactique facilitant l'activité lysosomiale de l'hôte en fragilisant la couche externe de la paroi bactérienne (Alakomi *et al.* 2000). Autre mécanisme est l'action de métabolites bactériens comme des acides gras à chaîne courte, l'acide lithocholic.

Enfin, certaines souches bactériennes (*Bacteroides*) peuvent activer les cellules dendritiques pour secréter localement des IgA (He *et al.* 2007).

6- Microbiote intestinal et fonctions digestives

a. Modèle des animaux axéniques

L'effet du microbiote sur le développement des constituants du tube digestif a été étudié initialement chez les animaux axéniques, c'est-à-dire exempts de flore. Ces animaux présentent des caractéristiques phénotypiques particulières, parfois réversibles après re-colonisation (Smith, McCoy and Macpherson 2007).

La paroi digestive est modifiée par le statut axénique (Figure 35) : les villosités intestinales sont plus petites, les cryptes moins profondes, le caecum est élargi, avec une paroi plus fine contenant des villosités irrégulières (Smith, McCoy and Macpherson 2007).

D'un point de vue fonctionnel, ont été constatées une baisse de la fréquence et de l'amplitude des contractions du muscle circulaire dans le jéjunum et l'iléon de souris (Collins *et al.* 2014). Une autre étude a montré que les modifications du régime alimentaire responsables d'une modification de la flore étaient capables de modifier le temps de transit intestinal (Figure 37) (Kashyap *et al.* 2013).

Concernant le SNE, des modifications morphologiques des neurones myentériques ont été décrites (Dupont, Jervis and Sprinz 1965) (Figure 36). Plus récemment ont été constatés au sein du plexus myentérique une densité de fibres nerveuses plus faible, une baisse de nombres de neurones par ganglions, une augmentation de la proportion de neurones nitrergiques (Collins *et al.* 2014). De plus, l'excitabilité des neurones sensitifs était diminuée, se corrigeant avec une colonisation normale du tube digestif (McVey Neufeld *et al.* 2013).

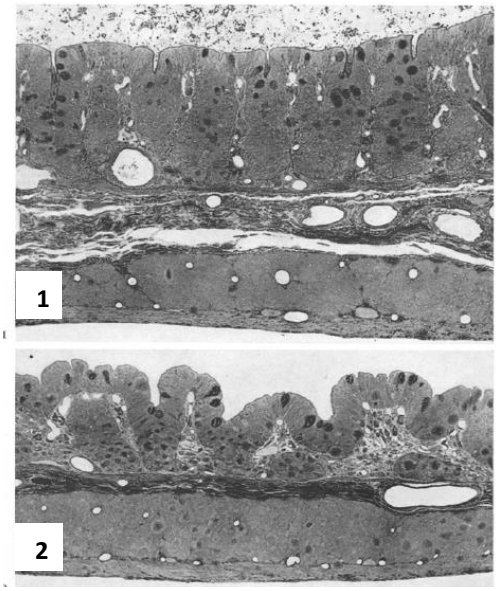


Figure 35 : Effets du microbiote sur la paroi intestinale.
Microphotographies de caecums de rats colonisés (1) et axéniques (2) montrant que le statut axénique est associé à des modifications structurelles de la paroi digestive (X230) (Gustafsson and Maunsbach 1971).

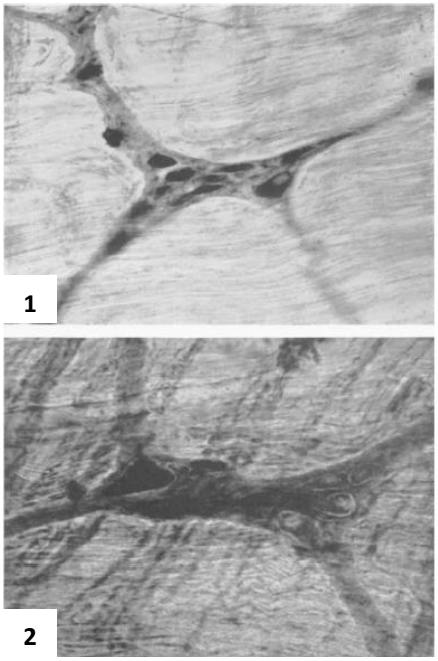


Figure 36 : Effets du microbiote sur les neurones entériques.
Microphotographies de ganglions entériques (DPNH diaphorase) du caecum de rats colonisés (1) ou axéniques (2). On remarque une modification de taille des neurones et une modification de l'intensité de la coloration (X300) (Dupont, Jervis and Sprinz 1965).

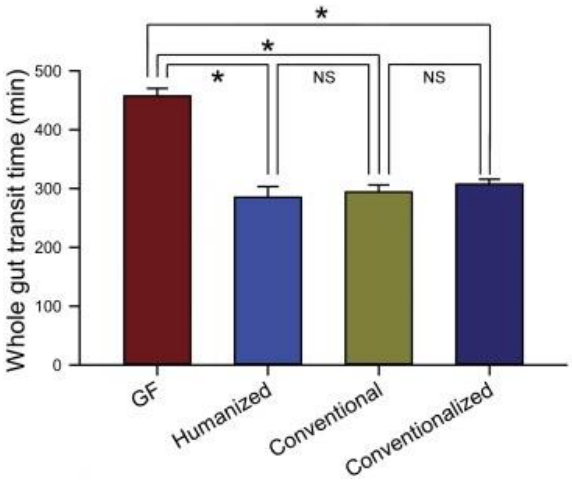


Figure 37 : Effets du microbiote sur le temps de transit total de la souris axénique.
Le temps de transit total (rouge carmin) est allongé chez les souris axéniques (GF) comparé aux souris « humanisées » (souris axéniques colonisées par flore humaine), aux souris conventionnelles et aux souris axéniques colonisées (conventionalized). (Kashyap et al. 2013).

b. Rôle de la flore sur la motricité digestive

Husebye a utilisé des rats axéniques afin de définir le rôle de certaines bactéries sur la motricité digestive. En colonisant le tube digestif par *Lactobacillus acidophilus* A10 et *Bifidobacterium bifidum* B11 la période des complexes moteurs migrants de l'intestin était accrue comme avec l'utilisation de *Clostridium tabificum* sp VP 04. Par contre, la colonisation de rats axéniques par *E.Coli* X7 avait l'effet inverse en augmentant la période des CMM (Figure 38). Dans une autre expérience, le temps de transit intestinal a été mesuré par index radio-actif (Figure 39). Celui-ci était plus rapide chez les animaux colonisés par une flore conventionnelle puis par *Lactobacilli acidophilus* et *Bifidobacterium bifidum* par rapport aux animaux axéniques (Husebye *et al.* 2001).

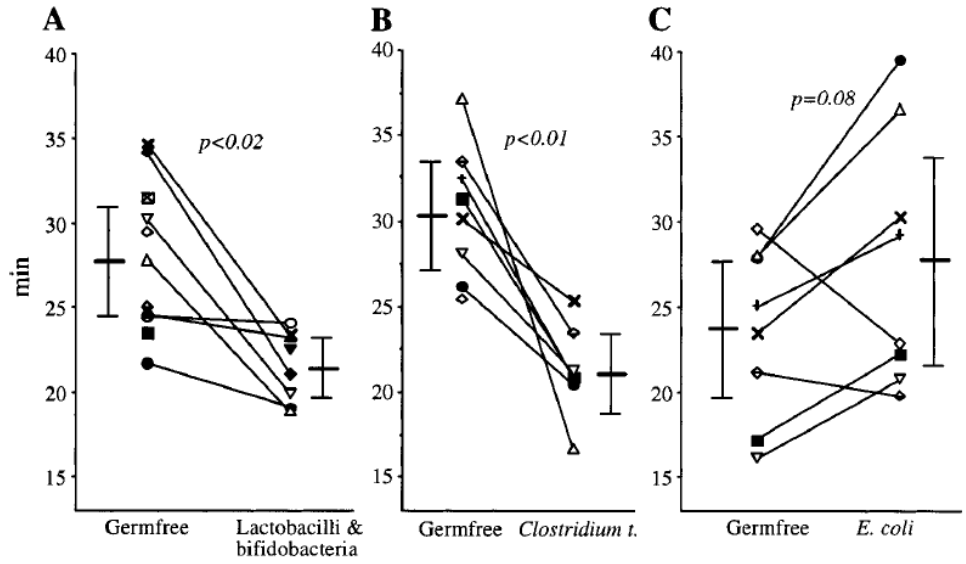


Figure 38 : Impact de souches bactériennes sur la motricité intestinale de rats axéniques.
La période des complexes moteurs migrants (CMM) a été mesurée *in vivo* sur l'intestin grêle de rats axéniques (germ free). Après contamination par *Lactobacillus acidophilus* A 10 et *Bifidobacterium bifidum* B11 ou *Clostridium tabificum* VP04 la période des CMM était diminuée. Elle était augmentée après contamination par *Escherichia Coli* X7 (Husebye et al. 2001).

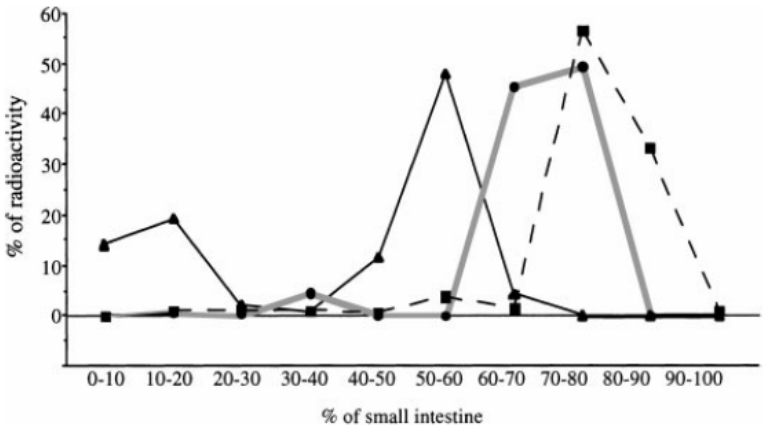


Figure 39 : Influence de souches bactériennes sur le temps de transit intestinal de rats axéniques.
Le temps de transit intestinal a été mesuré par index radio-actif : mesure du pourcentage d'intestin radio-actif 1 heure après gavage de marqueur radio-actif. Les rats axéniques (ligne noire) et les rats axéniques colonisés par *Lactobacilli acidophilus* A10 et *Bifidobacterium bifidum* B11 (ligne grise) ont un transit plus lent que les rats conventionnels (pointillé). (Husebye et al. 2001).

L'effet de bactéries vivantes sur la motricité digestive semble varier en fonction des souches utilisées et de l'organe étudié. La réponse motrice d'explants jéjunaux ou coliques de souris colonisées (non axéniques) a été étudiée par des cartes spatio-temporelles en (Figure 40) (Wu et al. 2013). L'application de Lactobacillus reuteri (DSM 7938) dans la lumière digestive a induit dans le jéjunum une baisse de fréquence et de vélocité des CMM, sans modification de la pression des pics de pression des CMM. Au niveau du côlon, il a été observé une augmentation de la fréquence et de la vélocité des CMM sans effet sur les pics de pression des CMM. De plus, *Lactobacillus salivarius* utilisé dans les mêmes conditions avait un effet différent sur le jéjunum (baisse de la fréquence et des pics de pression des CMM, augmentation de la véolicité des CMM) et sur le côlon (augmentation de fréquence et de la vélocité des CMM, sans effet sur les pics de pression des CMM).

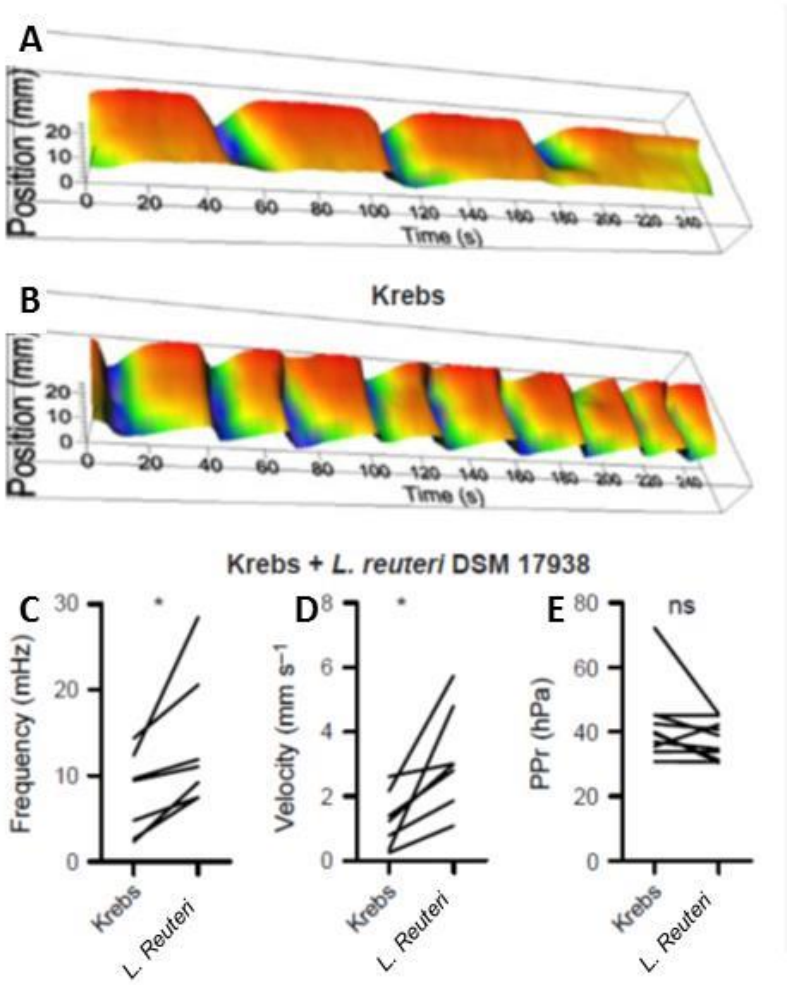


Figure 40 : Effets de *Lacobacillus Reuteri* sur la motricité d'explants coliques de souris.

Des explants coliques souris conventionnelles ont été prélevés, canulés et irrigués par une solution de Krebs sans (A) ou avec *Lacobacillus Reuteri* (B). L'explant colique a été filmé et le signal a été retranscrit en codage couleur (contractions : couleurs chaudes ; relaxations : couleurs froides). *Lacobacillus Reuteri* a permis une augmentation de la fréquence et de la vitesse des contractions spontanées coliques (C, D). La pression intraluminale du pic de pression (PPr peak phasic intraluminal pressure), mesurée par un cathéter intraluminal, était inchangée (E) (Wu et al. 2013).

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c. Rôle du microbiote dans le maintien de l'intégrité de la barrière épithéliale

Chez l'enfant, la supplémentation alimentaire en bactéries dites « probiotiques » a été testée dans de nombreuses pathologies qui sont associées à une augmentation de la perméabilité intestinale. Par exemple, les enfants porteurs de dermatite atopique ont été traités par *Lactobacillus rhamnosus* 19070-2 et *Lactobacillus reuteri* DSM 12246 permettant une diminution significative du ratio lactulose/mannitol après six semaines de traitement (Rosenfeldt et al. 2004). Chez l'enfant prématuré, l'apport de *Bifidobacterium Lactis* a permis également une baisse du ratio lactulose/mannitol au bout de sept jours de traitement, se prolongeant jusqu'à un mois de vie (Stratiki et al. 2007) (Figure 41). Dans ce contexte, une méta-analyse de la Cochrane data base récente incite ainsi l'usage de probiotiques (lactobacilles et/ou bifidobactéries) chez le prématuré dans le but de diminuer la survenue d'entérocolite ulcéro-nécrosante sévère (RR=0.43, [0.33 - 0.56]; 20 études, 5529 enfants), la baisse de mortalité globale (RR=0.65, [0.52 - 0.81]; 17 études, 5112 enfants) (AlFaleh and Anabrees 2014).

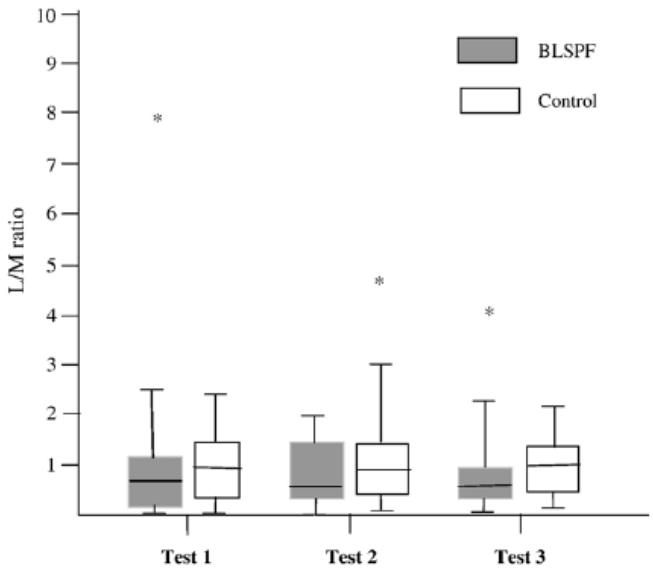

Figure 41 : Effet de souches de *Bifidobacterium Lactis* sur le ratio lactulose/mannitol chez le prématuré.
La supplémentation de *Bifidobacterium Lactis* (boîtes grises) dans l'alimentation de prématurés a permis une baisse de la perméabilité intestinale à 7 jours de vie (test 2) et à 30 jours de vie (test 3) (Stratiki et al. 2007).

Un des mécanismes impliqués serait le maintien de la cohésion épithéliale. Ainsi *Bacteroides thetaiotaomicron* a permis la production de small proline-rich protein 2A (sprr2A) renforçant l'action des desmosomes. *Escherichia coli Nissle 1917* a permis d'augmenter l'expression de ZO-2, favorisant une redistribution de ZO-2 du cytosol vers la membrane et d'augmenter l'expression de claudine 14 (Zyrek et al. 2007). Des métabolites secrétés par *Bifidobacterium infantis Y1* ont favorisé l'expression de ZO-1 et d'occludine et une baisse d'expression de claudin 2 augmentant la résistance transépithéliale (Corridoni et al. 2012). *Lactobacillus plantarum* MB452 a induit une augmentation de la transcription des gènes codant pour l'occludin et la cingulin (Anderson et al. 2010).

D'autres bactéries ont également montré un impact bénéfique sur l'intégrité de la barrière épithéliale telles que *Lactobacillus plantarum* (Karczewski *et al.* 2010), *Lactobacillus salivarius* UCC118 et CCUG38008, *Lactobacillus rhamnosus GG, Lactobacillus casei* DN-114 001, et *Lactobacillus casei Shirota* (Zakostelska *et al.* 2011; Agostini *et al.* 2012; Miyauchi *et al.* 2012; Wang *et al.* 2012; Wagnerberger *et al.* 2013).

Hypothèse et présentation des résultats

Ce travail de thèse s'intègre dans la compréhension des mécanismes impliqués dans la maturation post-natale du tube digestif et en particulier du système nerveux entérique. Il s'inscrit dans la continuité d'une thématique développée dans l'unité INSERM 913. En effet, des travaux antérieurs (de Vries *et al.* 2010) (en annexe), auxquels j'ai collaboré lors de mon master 2, ont montré chez le raton que le contrôle de l'activité motrice propulsive du côlon par le SNE apparaissait en période post-natale notamment par le biais des neurones myentériques cholinergiques et nitrergiques. Cette maturation étant probablement sous le contrôle de facteurs génétiques et environnementaux, nous avons voulu tester l'hypothèse selon laquelle le microbiote pourrait contribuer à la maturation du SNE et des fonctions digestives. En effet, le tube digestif, considéré comme stérile pendant la période prénatale est colonisé dès l'accouchement par des bactéries de l'environnement proche du nouveau-né qui constitueront le microbiote. Ces bactéries participeraient à la régulation de la maturation des fonctions digestives soit via des composés de leur paroi ou soit par la production de métabolites. Parmi les métabolites d'intérêt figurent les acides gras à chaîne courte et en particulier le butyrate qui présente une augmentation importante de sa concentration colique dans les premières semaines de vie. De plus, des travaux réalisés dans l'unité ont montré chez le rat adulte la capacité du butyrate à moduler le phénotype neurochimique des neurones myentériques cholinergiques associé à une augmentation de l'activité motrice propulsive du côlon (Soret *et al.* 2010). Néanmoins, la capacité des bactéries ou du butyrate à moduler la maturation du SNE et/ou les fonctions digestives reste à être démontré.

Nous avons donc émis l'hypothèse que le microbiote, par sa composition ou par les métabolites produits, pouvait contribuer à la modulation de la maturation post-natale du phénotype neuro-glial et fonctionnel du côlon.

Cette thèse est constituée de trois publications visant à répondre à cette hypothèse. Les travaux présentés ont été réalisés au sein de l'unité, impliquant des approches *in vivo*, *ex vivo*, de microbiologie et de culture cellulaire.

Dans une première approche (publication 1), nous avons évalué l'effet du butyrate sur la maturation post-natale du phénotype neurochimique du SNE et sur les fonctions digestives du raton. Une méthode de lavements coliques a d'abord été mise au point permettant de délivrer une substance dans l'ensemble du côlon. Puis l'effet de lavements de butyrate de sodium en période post-natale a été évalué chez le raton à la fois sur le phénotype neuro-chimique du SNE et sur la motricité colique testée *in vivo* et *ex vivo*. Nous avons montré que les lavements de butyrate (2.5 mM) induisaient une augmentation de la proportion de neurones cholinergiques et nitrergiques du plexus myentérique du côlon distal. Parallèlement, nous avons observé une diminution du temps de transit du côlon distal mesuré *in vivo* associé à une augmentation de la réponse musculaire du côlon après stimulation électrique du SNE *ex vivo*. Cette première publication a permis non seulement de mieux comprendre les mécanismes physiologiques de la mise en place de la motricité colique propulsive du raton mais aussi de fonder le rationnel scientifique quant à l'utilisation de ce modèle pour étudier l'impact du microbiote dans cette période développé par la suite dans la thèse. En effet ce modèle animal pourrait être utilisé afin de déterminer l'effet spécifique de souches bactériennes d'intérêt et d'explorer les mécanismes mis en jeu dans la modulation des effets observés.

Apres avoir identifié la capacité d'un métabolite bactérien à moduler la maturation post-natale du SNE nous avons voulu mettre en évidence 1) la capacité de bactéries à moduler le phénotype neuro-chimique du SNE et 2) la spécificité ou non de la réponse observée par des souches bactériennes de lactobacilles et de bifidobactéries. Afin de répondre à cette question, nous avons développé un modèle de co-culture de SNE primaire avec une monocouche de cellules épithéliales intestinales sur filtres Transwell® (publication 2). Ces travaux ont permis de mettre en évidence la capacité de souches bactériennes à moduler différentiellement et indépendamment de leur genre ou de leur espèce l'expression neuronale de la ChAT ou du VIP. Cette étude pourrait fonder la base d'une approche ciblée visant à restaurer un phénotype particulier en fonction d'atteintes neurochimiques de pathologies caractérisées.

Afin de valider plus avant cette dernière étude, nous avons caractérisé l'impact *in vivo* de bactéries vivantes sélectionnées à partir de cette approche *in vitro* sur la base de leur capacité à moduler spécifiquement les populations neuronales cholinergiques et VIPergiques (publication 3). Dans un premier temps, nous avons caractérisé les paramètres de croissance de souches bactériennes d'intérêt afin de les administrer en phase exponentielle de croissance pour optimiser l'inoculum bactérien. Nous avons ensuite mis en place une procédure d'administration orale de bactéries limitant le stress néonatal et avons étudié *in vivo* et *ex vivo* les fonctions de motricité et de perméabilité digestive. Un des résultats majeur de cette étude est que l'apport de bactéries a permis d'induire des modifications significatives à la fois des fonctions digestives et du phénotype neuro-glial du SNE. Cette approche renforce le concept de thérapeutique ciblée sur la restauration d'un phénotype et/ou d'une fonction dans le cadre de pathologies digestives caractérisées.

Résultats

1. Impact du butyrate sur la maturation du système nerveux entérique et les fonctions digestives

2. Impact *in vitro* du microbiote sur le phénotype du système nerveux entérique

3. Impact de souches bactériennes sur la maturation du système nerveux entérique et les fonctions digestives

1- Impact du butyrate sur la maturation du système nerveux entérique et les fonctions digestives

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

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

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Suply E, de Vries P, Soret R, Cossais F, Neunlist M. Butyrate enemas enhance both cholinergic and nitrergic phenotype of myenteric neurons and neuromuscular transmission in newborn rat colon. *Am J Physiol Gastrointest Liver Physiol* 302: G1373–G1380, 2012. First published April 5, 2012; doi:10.1152/ajpgi.00338.2011.—Postnatal changes in the enteric nervous system (ENS) are involved in the establishment of colonic motility. In adult rats, butyrate induced neuroplastic changes in the ENS, leading to enhanced colonic motility. Whether butyrate can induce similar changes during the postnatal period remains unknown. Enemas (Na-butyrate) were performed daily in rat pups between postnatal day (PND) 7 and PND 17. Effects of butyrate were evaluated on morphological and histological parameters in the distal colon at PND 21. The neurochemical phenotype of colonic submucosal and myenteric neurons was analyzed using antibodies against Hu, choline acetyltransferase (ChAT), and neuronal nitric oxide synthase (nNOS). Colonic motility and neuromuscular transmission was assessed in vivo and ex vivo. Butyrate (2.5 mM) enemas had no impact on pup growth and histological parameters compared with control. Butyrate did not modify the number of Hu-immunoreactive (IR) neurons per ganglia. A significant increase in the proportion (per Hu-IR neurons) of nNOS-IR myenteric and submucosal neurons and ChAT-IR myenteric neurons was observed in the distal colon after butyrate enemas compared with control. In addition, butyrate induced a significant increase in both nitrergic and cholinergic components of the neuromuscular transmission compared with control. Finally, butyrate increased distal colonic transit time compared with control. We concluded that butyrate enemas induced neuroplastic changes in myenteric and submucosal neurons, leading to changes in gastrointestinal functions. Our results support exploration of butyrate as potential therapy for motility disorders in preterm infants with delayed maturation of the ENS.

enteric nervous system; colonic motility; postnatal period

THE PERINATAL PERIOD IS A critical period of life during which major adaptive processes occur in various organs, such as the gastrointestinal (GI) tract. Indeed, although the GI tract is established early in embryogenesis, its maturation is still ongoing after birth. In particular, the intestinal mucosa shows important structural changes after birth (6), associated with changes in intestinal barrier functions such as paracellular permeability (33, 35). Moreover maturation of the immune system occurs during the postnatal period (30). In addition, these changes observed during this period also affect GI motility, as highlighted by studies performed in preterm infants.

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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 antroduodenal 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 motorneurons contain acetylcholine and substance P that induce smooth muscle contraction, whereas inhibitory motorneurons 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 litters) were kept in the same conditions during the whole experiments. 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 introduced in the rectum up to 5 mm, and a volume of 20 μl/g of animal weight was injected. This volume of enema was determined in preliminary experiments in PND 7 and PND 17 rats (n = 3). Methylene blue enemas (10% diluted in PBS) of 10, 20, and 100 μl/g of animal weight were tested. Whereas 100 μl/g reached the small intestine and 10 μl/g did not reach the cecum, 20 μl/g was able to reach the entire length of the colon (Fig. 1, *A* and *B*). We also showed that, at PND 21, 20 μl/g of methylene blue stained the entire length of colon. Enemas were injected slowly (10 s per enema), the cannula was then removed, and pups were maintained muzzle down during 20 s.

Enemas of mannitol (5 mM; Aguettant, Lyon, France), saline (NaCl 0.9%), sodium butyrate (10.0, 5.0, 2.5 mM; Sigma Aldrich, Saint Quentin Fallavier, France), or sham enemas (i.e., only insertion of the cannula) were performed.

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-diameter 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% CO_2-95% O_2) Krebs solution containing (in mM) 117.0 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 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)

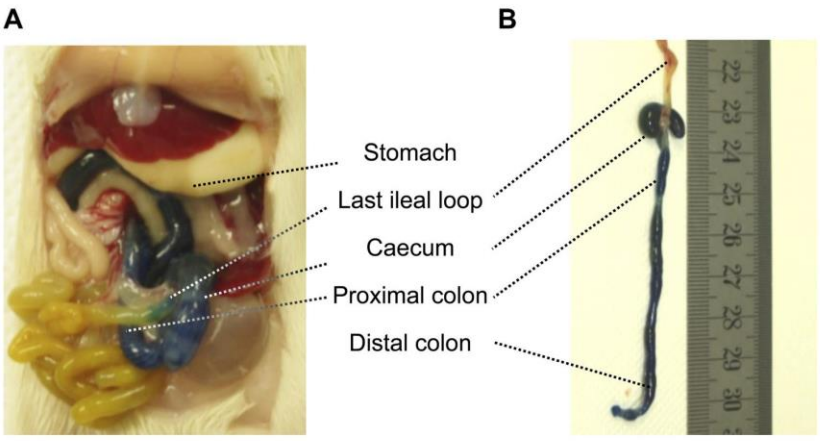

Fig. 1. Picture showing the distribution of methylene blue along the colon following an enema (20 μl/g) performed in a 17- day-old postnatal rat (*A*). Methylene blue stained the entire colon from the rectum to the cecum (*B*).

inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME, 50 mM, Sigma), and further in presence of atropine (10^{-6} M, Sigma), an antagonist of cholinergic muscarinic receptors. Drugs were applied 15 min before EFS. Tension level, amplitude of spontaneous contractions, and area under the curve (AUC) during each EFS-induced response were measured. Data were normalized to the weight of the tissue.

Paracellular Permeability Measurements in Ussing Chambers

Full-thickness segments of distal colon were mounted in 2-mm-diameter Ussing chambers (Easy Mount; Warner Instrument, Hamden, CT). Tissues were maintained at 37°C in DMEM (Invitrogen) containing 0.1% fetal calf serum (AbCys, Paris, France) continuously oxygenated (5% CO_2-95% O_2). After 30 min of equilibration, 200 μl of apical medium was replaced by 200 μl of sulfonic acid solution (578 Da, final concentration: 0.1 mg/ml) (Invitrogen). The fluorescence level in the basolateral chamber was measured every 30 min during 150 min using a fluorometer (Varioskan; Thermo Fisher Scientific, Courtaboeuf, France). The slope of the fluorescence intensity over time was determined by using a linear regression fit.

Immunofluorescence Attaining

Segments of proximal colon (1 cm adjacent to the cecum) and distal colon (1 cm, directly above the segment used in motility studies) were opened along the mesentery, pinned in Sylgard (Dow Corning, Midland, MI)-coated Petri dish, and fixed in 0.1 M PBS containing 4% paraformaldehyde (PFA) at room temperature for 3 h. Whole mounts of submucosal plexus and myenteric plexus were obtained following microdissection of the mucosa and the circular muscle.

Whole mounts were first permeabilized with PBS, 0.1% sodium azide, 4% horse serum, and Triton X-100 for 3 h at room temperature. Tissues were then incubated sequentially with primary antibodies for 16 h and the antibodies for 3 h in the following order: goat anti-choline acetyltransferase (ChAT) (1:200; Millipore, Billerica, MA) and anti-goat Cy3 (carboxymethylindocyanine) (1:500; Jackson ImmunoResearch, Suffolk, UK), rabbit anti-neuronal NOS (nNOS) (1:1,000; Alexis Laboratories, San Diego, CA) and anti-rabbit Cy5 (7-amino-4-indodicarbocyanin) (1:500, Jackson ImmunoResearch), and mouse anti-HuC/HuD (1:200, Invitrogen) and anti-mouse FITC (1:500, Jackson ImmunoResearch).

Specimens were viewed under an Olympus IX 50 fluorescence microscope fitted with adequate filter cubes. Pictures were acquired with a digital camera (model DP 71; Olympus, Rungis, France) coupled to the microscope. The numbers of Hu-, ChAT- and nNOS-immunoreactive (IR) cells were counted in at least 20 ganglia per condition. Myenteric ganglia were defined under the microscope as entities containing Hu-IR cells separated by a gap clearly distinguishable (about the size of one neuron or even smaller). Structures not clearly identified as ganglia were not analyzed. Data are expressed as 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.

Morphological Analysis

Pellet-free segments of distal colon were fixed in 4% PFA solution for 3 h at room temperature and embedded in paraffin. Sections were stained with hematoxylin and eosin. Measurements of the longitudinal and the circular muscle thickness, the height of crypts, and the mucosal and submucosal thickness were performed on five distinct fields of view from five animals in each condition as previously described (44).

Acetylcholine Assay

Pellet-free tubular segments of distal colon were placed in RIPA buffer (Millipore) and frozen at −80°C. Acetylcholine concentration was determined in tissue homogenates (Amplex Red, acetylcholine/acetylcholinesterase assay kit, Invitrogen) and normalized to the neuron-specific enolase (NSE) level (Prolifigen NSE IRMA; DiaSorin, Stillwater, MN).

Statistical Analysis

Data were 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. 2*A*). In addition, enemas of 5 mM but not 10 mM of butyrate also significantly reduced the bead latency time ($P < 0.001$) (Fig. 2*A*). However, bead latency time was not modified after saline, mannitol, or sham enema compared with unmanipulated animals (Fig. 2*B*). 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

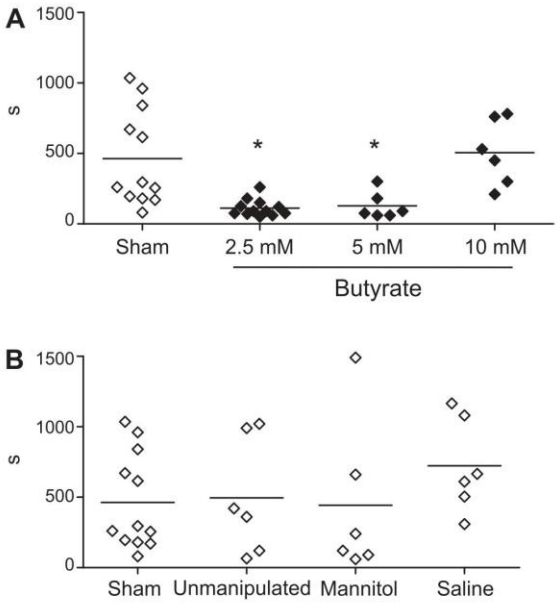

Fig. 2. Impact of butyrate enemas on bead latency. Butyrate enemas (2.5 mM; $n = 12$ and 5 mM; $n = 6$) reduced the expulsion time of a bead inserted in the rectum compared with controls (sham enemas; $n = 12$). In addition, enemas of 10 mM butyrate ($n = 6$) did not reduce the bead latency time compared with controls (*A*) (Kruskal-Wallis test followed by Dunn's multiple-comparison test; $^*P < 0.05$ compared with controls). Mannitol (5 mM), saline, and sham enemas did not modify the bead latency time compared with unmanipulated animals (*B*).

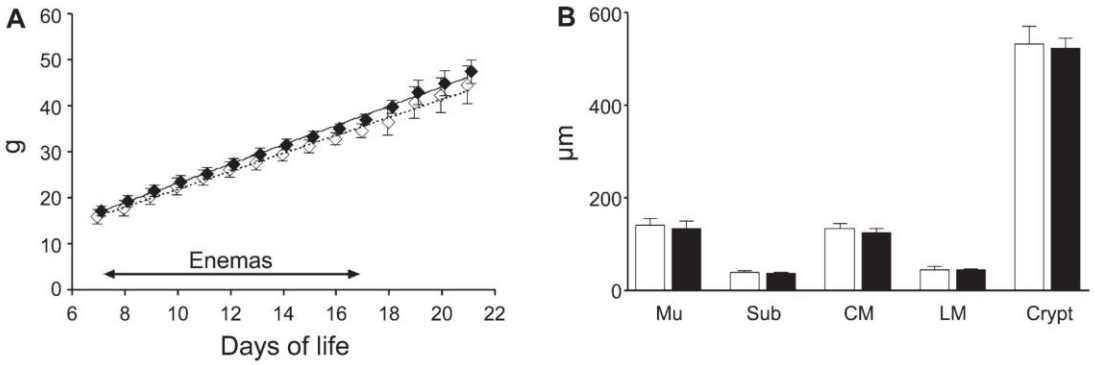

Fig. 3. Impact of butyrate enemas on weight gain and morphological parameters. Butyrate enemas (●) did not modify the weight of rats during the course of experiments compared with controls (sham enemas) (○) ($n = 8$) (*A*). Butyrate enemas (●) did not modify histological parameters of colon compared with controls (sham enemas) (○) ($n = 5$) in terms of thickness of mucosa (Mu), submucosa (Sub), circular muscle layer (CM), longitudinal muscle layer (LM), and height of crypts (Crypt) (*B*).

from controls (Fig. 3*A*). 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. 3*B*).

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

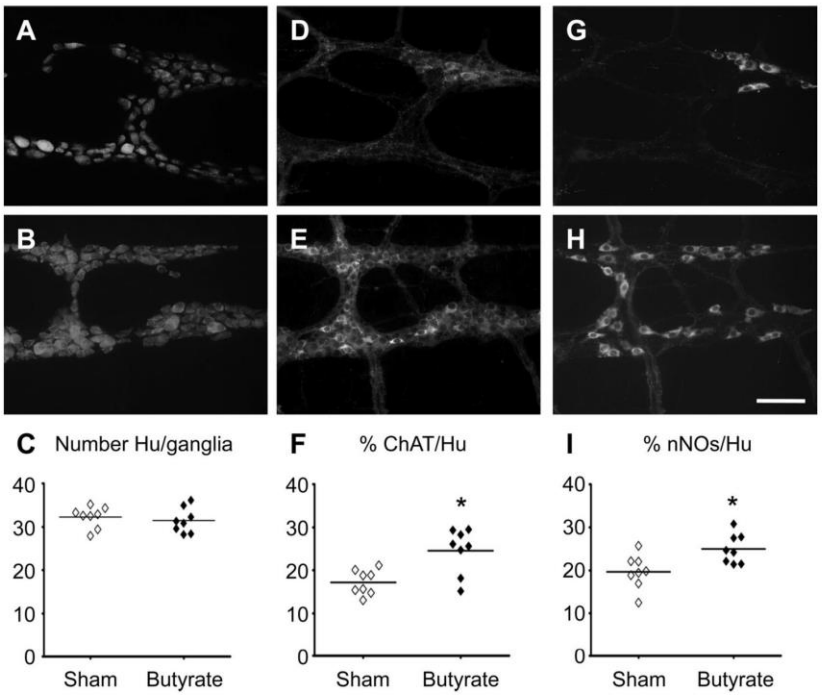

Fig. 4. Impact of butyrate enemas on neurochemical coding of myenteric neurons. Triple immunochemical staining of whole-mount preparations of myenteric plexus and longitudinal muscle layer. Immunochemical staining with antibodies against Hu revealed that butyrate enemas (*B* and *C*) did not modify the number of neurons per ganglia compared with controls (sham enemas) (*A* and *C*) ($n = 8$). Immunochemical staining with antibodies against choline acetyltransferase (ChAT) revealed that butyrate enemas (*E* and *F*) increased the number of ChAT-immunoreactive (IR) neurons per Hu-IR neurons compared with control (sham enemas) (*D* and *F*) (*$P < 0.05$; Mann-Whitney *U*-test; $n = 8$). Immunochemical staining with antibodies against neuronal nitric oxide synthase (nNOS) revealed that butyrate enemas (*H* and *I*) increased the number of nNOS-IR neurons per Hu-IR neurons compared with controls (sham enemas) (*G* and *I*) (*$P < 0.05$; Mann-Whitney *U*-test; $n = 8$) (scale bar = 100 µm).

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 Neuromuscular Transmission

Ex vivo motility studies were performed on segments of colonic longitudinal muscle. Basal tension level was similar between butyrate-treated animals compared with controls (2.6 ± 0.5 vs. 3.0 ± 1.4 mN/g of tissue, respectively; $n = 6$). In presence of L-NAME, the basal tension was increased in butyrate-treated animals (4.0 ± 0.6 mN/g of tissue; $n = 6$; $P = 0.03$) but not in controls (3.2 ± 0.7 mN/g of tissue; $n = 6$). In the presence of atropine, the basal tension decreased in both controls and butyrate-treated animals (2.2 ± 0.6 mN/g of tissue; $n = 6$; $P = 0.03$ and 2.7 ± 0.6 mN/g of tissue, respectively; $n = 6$; $P = 0.03$).

Amplitudes of spontaneous contractions were lower in the control group compared with butyrate (0.4 ± 0.3 vs. 0.9 ± 0.1 mN/g of tissue, respectively; $n = 6$; $P = 0.02$). In presence of L-NAME, amplitudes of spontaneous contractions were increased in butyrate-treated animals (1.3 ± 0.2 mN/g of tissue; $n = 6$; $P = 0.03$) but not in controls (0.4 ± 0.05 mN/g of tissue; $n = 6$). In the presence of atropine, amplitudes of spontaneous contractions tended to decrease in butyrate-treated animals (0.9 ± 0.3 mN/g of tissue; $n = 6$; $P = 0.053$) but not in the control group (0.5 ± 0.2 mN/g of tissue; $n = 6$).

Segments of colonic longitudinal muscle were stimulated by EFS, and EFS-induced contractile responses were then analyzed in the absence or presence of L-NAME and/or atropine (Fig. 5*A*). The EFS-induced AUC was larger in butyrate-treated animals compared with controls ($n = 6$; $P = 0.01$) (Fig. 5*B*). In the presence of L-NAME, EFS-induced AUC was significantly increased in both control group ($n = 6$; $P = 0.03$) and butyrate group ($n = 6$; $P = 0.02$) (Fig. 5*B*). However, the amplitude of L-NAME-sensitive AUC was significantly larger after butyrate treatment compared with controls ($n = 6$; $P = 0.01$) (Fig. 5*C*). In the presence of atropine, EFS-induced AUC was significantly decreased only in the butyrate-treated group ($n = 6$; $P = 0.02$) (Fig. 5*B*). Furthermore, the amplitude of atropine-sensitive AUC was higher in the butyrate group compared with controls ($n = 6$; $P = 0.03$) (Fig. 5*D*).

Impact of Butyrate On the Distal Colonic Permeability

We next determined whether butyrate could modify the paracellular permeability in the distal colon. Sulfonic acid flux across the colonic mucosa was identical in butyrate-treated animals compared with controls (Fig. 6*A*). 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. 6*B*).

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

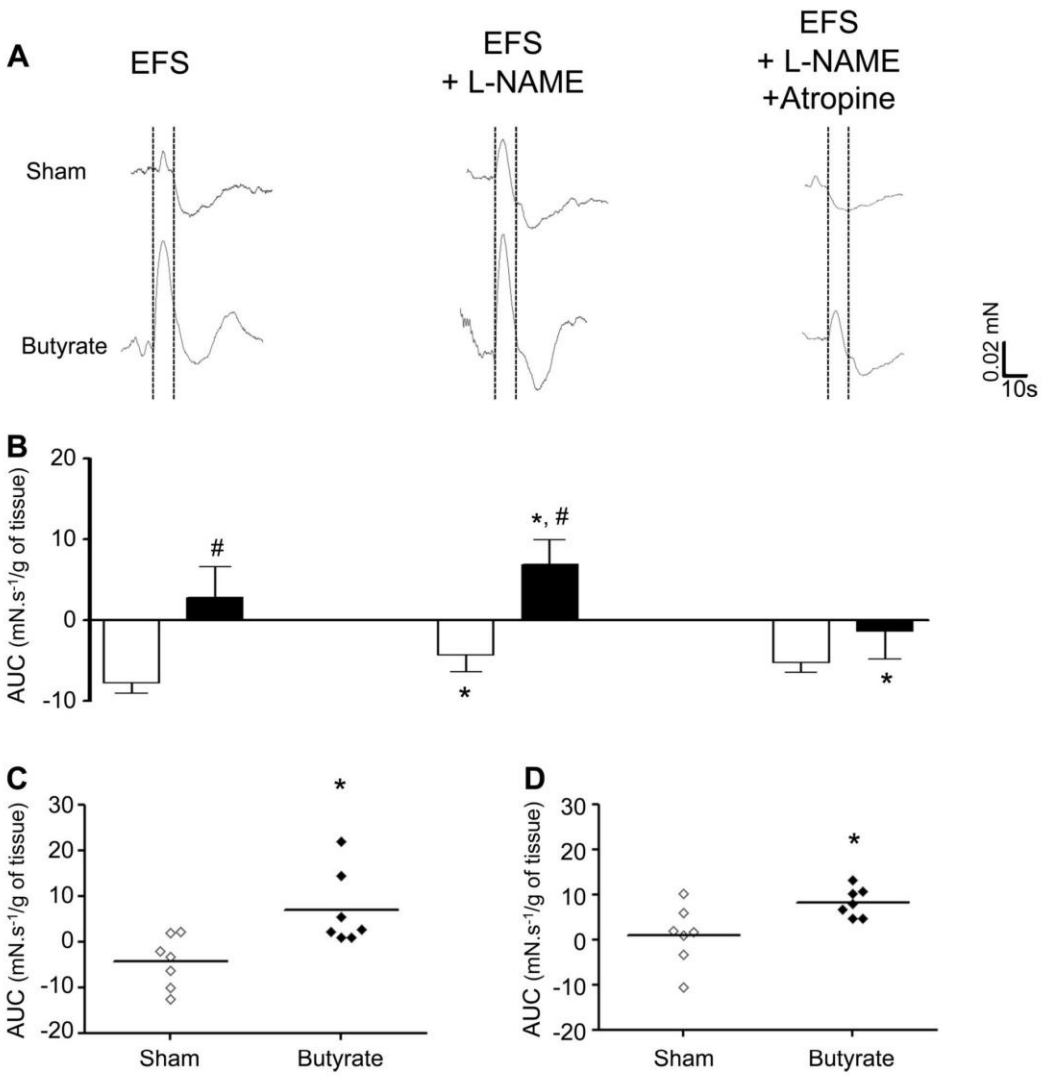

Fig. 5. Impact of butyrate enemas on neuromuscular transmission assessed ex vivo. Distal colonic longitudinal muscle segments were stimulated by electrical field stimulation (EFS). The area under the curve (AUC) of EFS-induced contractile response was analyzed in absence or in presence of *N*-nitro-L-arginine 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; $n = 6$).

(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

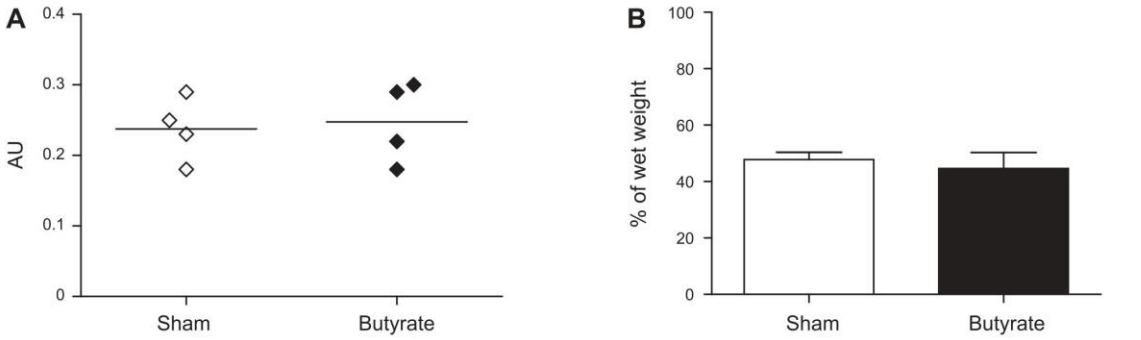

Fig. 6. Impact of butyrate on colonic permeability. Paracellular permeability assessed ex vivo in Ussing chambers and sulfonic acid flux were not modified in the butyrate-treated group compared with controls (sham enemas) (*A*). The water content of feces was unchanged after butyrate enemas compared with controls (sham enemas) (*B*).

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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2- Impact *in vitro* du microbiote sur le phénotype du système nerveux entérique

Titre : Comparative analysis of induction of neuroplastic changes in the enteric nervous system by a highly diverse set of *Bifidobacteria* and *Lactobacilli*.

Auteurs : Margarida Ribeiro Neunlist, Mandy Birau, Sandrine Capronnier, Pascale Rondeau, Etienne Suply, Gianfranco Grompone, Biliana Lesic, Michel Neunlist

Objectifs : tester l'effet de souches bactériennes sur le phénotype neurochimique du SNE et sur la barrière intestinale sur un modèle *in vitro.*

Implication personnelle : j'ai participé à l'analyse des données et à la sélection des souches pour le modèle *in vivo*.

Valorisation : ce travail a fait l'objet d'une communication orale longue à l'UEGW.

Comparative analysis of induction of neuroplastic changes in the enteric nervous system by a highly diverse set of *Bifidobacteria* and *Lactobacilli*.

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Abstract

Background

Alterations in enteric nervous system (ENS) and barrier integrity are frequently associated to functional bowel disorders. Nutritional factors, in particular bacteria-derived metabolites, have profound impact upon the phenotype of the ENS and its regulation of gastro-intestinal functions. However, the ability of probiotics to regulate neuromediator expression in the ENS remains largely unknown. Therefore, we aimed to explore the impact of diverse set of Lactobacillus and Bifidobacterium strains isolated from humans or (fermented) foods on the ENS and simultaneously, on intestinal barrier permeability.

Methods

49 Bifidobacteria and 20 Lactobacilli were inoculated on a co-culture model composed of primary culture of rat ENS and confluent colonic human T84 cells seeded on Transwell® filters. The expression of vasoactive intestinal peptide (VIP) and choline acetyltransferase (ChAT) were assessed by immunoassay and immunohistochemical analysis, respectively in primary culture of ENS. Additionally, in a separate set of experiments, the effect of strains on barrier integrity was addressed by measuring changes in transepithelial resistance (TER) of confluent T84 cells after incubation with each strain.

Results

Modulation of neuronal ChAT expression was induced by 35% of Bifidobacteria strains and 25% of Lactobacilii strains. ChAT expression was increased by 8% of Bifidobacteria strains and decreased with 27% of other Bifidobacteria strains. In contrast, Lactobacilli strains induced only an increase in ChAT expression. VIP expression was modulated by 10% of each genus (5% up and 5% down). Only one Bifidobacterium bifidum (BB80 strain) was able to significantly increase VIP and decrease ChAT expression. Interestingly, this strain also showed the capacity to increase TER.

Conclusion

We have developed and validated an *in vitro* coculture model allowing the study of bacterial strains effects upon key neuromediators expression in the ENS. Our study identified that Bifidobacteria and Lactobacilli could differentially modulate the neurochemical phenotype of ENS in a strain-dependent manner. This approach could be used to explore the molecular and cellular mechanisms of bacteria-ENS interactions but also to identify strains with putative beneficial or therapeutic effects upon the ENS and GI functions.

Introduction

The enteric nervous system (ENS) is an integrated nervous system organized all along the gut into two major plexus with functional specific roles (Furness, 2013). The myenteric plexus, located between the longitudinal and circular muscle, contains neurones mainly involved in the control of gastrointestinal (GI) motility. The submucosal plexus, located between the circular muscle and the mucosa, contains neurones mainly involved in the control of intestinal epithelial barrier (IEB) functions (Neunlist et al., 2013).

The IEB is constituted by a continuous monolayer of polarized specialized cells that are held together by desmosomes, adherent junctions and tight junctions. Tight junctions regulate the paracellular passage of particles, whereas adherent junctions and desmosomes are strong adhesive bonds between intestinal epithelial cells that confer mechanical strength to the IEB (Machiando et al., 2010). *In vitro* and *in vivo* studies have shown that enteric neuromediators can differentially regulate intestinal paracellular permeability (Hällgren et al., 1998, Hardin et al., 1999, Hayden et al., 2000; Neunlist et al., 2003). For instance, enhanced neuronal synthesis of acetylcholine in the colon leads to an increase of epithelial paracellular permeability in rats (Gareau et al., 2007). In contrast, vasoactive intestinal peptide (VIP) released by submucosal neurons reduces paracellular permeability in part by upregulating the expression of tight junction protein ZO-1 in intestinal epithelial cells (Neunlist et al., 2003). Further reinforcing the role of VIP in enhancing barrier function is a recent study showing that VIP increased barrier resistance to *Citrobacter rodentium* induced colitis in mice (Conlin et al., 2009).

Reinforcing IEB resistance and reducing paracellular permeability could be benefit in various gastrointestinal disorders such as irritable bowel syndrome (IBS) or inflamamtory bowel diseases. Indeed, increased intestinal paracellular permeability or altered expression of tight junctions proteins has been reported in IBS patients as compared to healthy controls and has been positively correlated with visceral pain (Piche et al., 2009; Zhou et al., 2009; Annahazi et al., 2013) Similarly, increased paracellular permeability has been shown to precede relapse or is increased in IBD relatives (Fries et al., 2013). Demonstrating the causal role of increased permeability in IBS and/or IBD are studies showing that reducing barrier permeability using chemical tight junction blockers or zonulin antagonists reduced pain score and inflammation in animal models of IBS (Ait Belgnaoui et al., 2005) and IBD (Arieta et al., 2009), respectively. Therefore, approaches aiming at modulating the expression of neuromediators involved in regulation of intestinal barrier permeability could be of benefit in the treatment of IBS.

Luminal factors (such as nutrients, microbiota) are increasingly recognized as new approaches to modulate the phenotype and functions of enteric neurons and ultimately gut functions that they regulate (Neunlist and Schemann, 2014). For instance, recent studies have shown that bacterial metabolites such as short chain fatty acids can increase the expression of ChAT in myenteric neurons which in turn leads to an increase of colonic motility both in adult and newborn rat (Soret et al., 2010; Suply et al., 2012). Other nutritional factors such as saturated or polyunsaturated lipids can also profoundly impact upon neuronal phenotype and/or survival (de Quelen et al., 2011; Baudry et al., 2012). However, currently, there are limited evidences that commensal or probiotic bacteria can modulate the neuromediator expression in the ENS although probiotic can induce changes in electrophysiological properties of enteric neurons (Saulnier et al., 2013). Besides demonstrating the ability of microbiota to modulate enteric neuronal and gut functions, these studies also demonstrated strains specific changes. For instance, *L. reuteri* enhanced excitability of myenteric primary sensory (AH) neurons and concomitantly inhibited motility (Wang et al., 2010). In contrast, *Bifidobacterium longum* NCC3001 reduced excitability of AH neurons (Khosdel et al., 2013).

Concerning the modulation of ENS phenotype, *Saccharomyces cerevisiae sp boulardii* was shown to increase the expression of Calbindin-immunoreactive (IR) neurons (Kamm et al, 2004). In addition, *Pediococcus acidilactici* increased the number of galanin- and calcitonin gene related peptide (CGRP)-IR neurons in the submucosal plexus of the ileum (di Giancamillo et al, 2010). However, the impact of bacterial strains upon the regulation of neuromediators involved in the control of intestinal barrier permeability such as VIP or ACh remains currently unknown. In this context, the aim of this study was to determine if bacterial strains such as Bifidobacteria and Lactobacilli were able to influence the phenotype of enteric neurons and IEB resistance. This objective was achieved using an integrated *in vitro* coculture model composed by primary culture of ENS and a monolayer of intestinal epithelial cells incubated with specific strains developed by Moriez *et al*. (2007). Simultaneously, the same set of bacteria was assessed for their capacity to directly modulate the intestinal epithelial resistance, using a model previously described (Miyauchi et al, 2009, Commane et al.,2004).

Materials and methods

Bacterial strains and growth conditions

This study was performed with 69 strains provided by Danone Research that belonged to two genera: Lactobacillus and Bifidobacterium *(Supplemental data. Table 1)*. Lactobacilli were grown in Man, Rogosa and Sharpe medium (MRS) (Biokar Diagnostics, Beauvais, France). Bifidobacteria were cultured in a defined medium which consisted of a tryptone peptone (Becton Dickinson-USA) basis supplemented with glucose (Sigma, France), yeast extract (Becton Dickinson-USA) and hemine (TGYH medium, Calbiochem, France). Bacterial stock cultures were maintained in cryobeads (Cryogene), stored at -80°C.

For all experiments, the final culture consisted of 100mL of growing medium inoculated at 1/10 (*Bifidobacterium*) or 1/100 (*Lactobacillus*) with a specific overnight pre-culture. Lactobacilli were cultured for 24h at 37°C under air atmosphere conditions while Bifidobacteria were cultured for 24 or 48h, as function of strain, at 37°C under anaerobic conditions (AnaeroGen^{TM}, Oxoid, England). Pureness of bacterial cultures was assessed by microscopic observation of methylene blue (Réactifs Ral, Martillac, France) stained slides as well as plate spreads.

Primary enteric nervous system and intestinal epithelial cell cultures

Primary cultures of rat ENS were obtained from fifteen days foetuses as previously described (Chevalier et al., 2008). In brief, pregnant Sprague Dawley rats (CERJ, Le Genest St Isle, France) were euthanized by a CO_2 overdose and their uterus were removed. Foetus intestines were subsequently dissociated and processed using a protocol using trypsin (Sigma, T1426) and Dnase (Sigma DN25) reactions. Transformed human intestinal epithelial cells T84 were obtained from the ATCC collection (CCL-248^{TM}). T84 cells were seeded in 12-well Transwell® filters (Corning, NY, USA) at a density of 2×10^5 cells/well and cultured for 17 days to obtain confluence in Dulbecco's modified Eagle medium (DMEM-F12; 1:1; GIBCO) supplemented with 10% heat-inactivated FBS and 50 IU/ml penicillin, and 50 µg/ml streptomycin. All cells were maintained in incubators (95% air, 5% CO_2) at 37°C.

Co-culture model of enteric nervous system primary cultures and T84 cells

The co-culture model was adapted from a model developed in our laboratory (Moriez et al., 2009). 24 hours prior to the establishment of the co-culture, T84 culture medium was replaced by primary ENS medium. Co-culture experiments were performed by culturing primary ENS cultures with Transwell of T84 treated with bacteria.

The optical density (OD) of bacterial cultures was measured by spectrophotometry (BioSpec-mini, Avantec, France, wavelength of 580 nm) to calculate the adequate volume of culture to centrifuge to obtain the adequate CFU (4000 rpm, at room temperature for 15 min). The supernatant was discarded, and the cell pellet was washed in phosphate buffered saline (PBS) and resuspended in an appropriate volume of primary ENS culture medium (DMEM-F12 1:1, containing 1% of N-2 supplement (Life Technologies, Cergy Pontoise, France). Eight hours later, Transwell filters containing bacteria, were discarded and primary ENS culture was maintained for 22 hours in the incubator (95% air, 5% CO_2) at 37°C. In order to analyse primary ENS culture by immunohistochemical (IHC) and enzyme immunoassay analysis (EIA), two identical sets of co-culture were performed systematically.

Each experiment included 2 positive controls with KCl (40mM; Sigma) inducing VIP expression and with sodium butyrate (500µM; Sigma) inducing ChAT expression in ENS primary culture as previously described (Soret et al., 2010; Chevalier et al., 2008). For negative control ENS primary cultures were cultured with T84 alone. Each bacterial strain was assessed at least in triplicate. Positive and negative controls were assessed in duplicate in each microtiter plate.

Immunohistochemistry and identification of neuronal cell populations

At the end of the co-culture experiments, one of the two identical sets of primary ENS culture was fixed in a 4% paraformaldehyde-PBS suspension and processed for IHC studies as previously described (Chevalier et al., 2008). Cells were simultaneously incubated with primary antibodies goat anti-ChAT (1:150; Millipore, France) and mouse anti-HuC/D (1:200; Molecular Probes, OR, USA) for 90 minutes. After three consecutive washes in PBS, cells were incubated with secondary antibodies CY3-conjugated affinity pure donkey anti-goat IgG (1:500; Jackson Immunoresearch, BA, USA) and FluoProbes 488-conjugated anti-mouse IgG (1:200; FluoProbes®, France) for 30 minutes and again washed three times in PBS.

The number of ChAT-immuno-reactif (IR) and Hu-IR cells was counted in at least 20 ganglia per well (average of 389.7 ± 60.1 neurones per preparation) and their ratios (% ChAT) calculated. Results were normalized and expressed as the average of the ratios between % $ChAT_{(strain)}$ and % $ChAT_{(T84)}$ (negative control).

Protein analysis

The second set of primary ENS cells was used to prepare protein extracts for further quantification of neuron specific enolase (NSE) as well as VIP. Primary ENS cells supernatant was recovered and stored at -80°C until extracellular NSE analysis. For determination of intracellular NSE and VIP, the intracellular proteins were extracted using RIPA lysis buffer (Millipore, Billerica, MA, USA) containing a protease inhibitor cocktail (Roche Diagnostics, Meylan, France). Extractions were stored at -80°C until intracellular VIP and NSE assay.

Extracellular NSE (NSE_{ext}) and intracellular NSE (NSE_{int}) were measured using an immunoradiometric kit (Diasorin, Stillwater, USA). NSE release in the culture medium was calculated using the ratio $3NSE_{ext}/(3NSE_{ext}+NSE_{int})$ since suspensions characterized by volumes of 3×500µL and 500µL were used to quantify NSE_{int} and NSE_{ext} respectively. Intracellular VIP was quantified using enzyme immunoassay kit (Peninsula laboratories, Bachem, SA). VIP results were normalized to NSE_{int} in order to account for the differences in number of neurones per well.

Results were normalized and expressed as the ratios between (VIP_{int}/NSE_{int}) strain and (VIP_{int}/NSE_{int}) T84 (negative control).

Transepithelial resistance (TER) assay

T84 cells were cultured in Dulbecco's modified Eagle medium (DMEM/F12; Invitrogen) supplemented with 20 % FCS (foetal calf serum), 1 % Essential Amino Acid (100X) (Invitrogen) and penicillin (100 U/ml) /streptomycin (0.1 mg/ml) (Invitrogen).

For growth on porous filters, 10^5 T84 cells were plated on Transwell filters (0.4 µm pore size Corning® HTS Transwell®-12 wells) and incubated at 37°C for 10 days in a 5% CO_2 incubator. The TER of filter-grown T84 cell monolayers was measured using a volt ohmmeter (EVOM2). After 10 days, the initial TER (Ω) was measured (T0) and plate used if TER value ranged within 900-1400 Ω. Bacteria (Lactobacilli or Bifidobacteria) washed and resuspended in PBS were subsequently added on the apical side at a multiplicity of infection

of 40. After 4h of incubation at 37°C in a 5% CO_2 incubator, TER was measured and the ratio T4h/T0h calculated. Each bacterial strain was tested in triplicate on each microtiter plate and in at least three independent experiments. On each microtiter plate the untreated controls (PBS) was included in triplicate.

Statistical analysis

For the neuronal model, the 69 strains were evaluated randomly over 34 runs of experiments. Negative (T84 without bacteria) and positive controls were included in each run. In each run, two or three repetitions were carried out with strains and controls. Each strain was evaluated with two or three independent bacterial cultures. All data coming from the control were used to evaluate the accuracy of the model. The outlier values were detected on raw data using the accuracy characteristics of the model (repeatability and reproducibility). At each run, ChAT and VIP values were normalized by the value of the corresponding control T84. The means by strain and by run were calculated and used in an ANCOVA model (with T84 value as baseline) to determine the effect of strain on the biological responses. If the p value associated to the strain was equal or less to 5%, the strain effect was judged significant; between 5 and 10% a tendency was considered. Due to the exploratory context of the study no adjusted p value procedure was applied. A variability chart taking account genus and species was done for each biological response to illustrate the source of variance of the biodiversity. Pearson and spearman correlations were done to explore the link between the different parameters.

For the TER model, the 69 strains were evaluated randomly over 29 runs of experiments. In each run, strains and negative control were tested in triplicate and each run was repeated at least three times within dependent bacterial cultures. All data coming from the control were used to evaluate the accuracy of the model. The ratio TER value after 4 hours divided by the TER value at baseline, were calculated for each strain and control; by experiments and by run. The repeatability and reproducibility of the experiments were evaluated, the variance was found to be below 10%. An ANOVA test followed by a Dunnett test was used to evaluate the effect of the strain on the TER response, and to compare the effect of the strain to the control effect. If the p value associated to the strain is equal or less to 5%, the strain effect was judged significantly different from the control; between 5 and 10% a tendency was considered.

Results

Validation of the co-culture model with ENS primary cultures and T84 cells

In a first step we aimed to determine the sensitivity and reproducibility of our model to detect changes in our populations of interests i.e. cholinergic and VIPergic neurons. We used two "model stimuli" previously shown to increase ChAT and VIP expression in primary culture of ENS, i.e. sodium butyrate and KCl, respectively.

Every co-culture experiment comprised the positive controls (ENS cells incubated with either sodium butyrate or KCl) as well as negative controls (ENS cultured with T84 alone). The cholinergic and VIPergic phenotype associated to these controls is shown in Table 1. In presence of butyrate, the proportion of ChAT neurons was higher than in ENS cultured with T84 alone, 58.1 ± 4.0% and 43.5 ± 3.1% of ChAT neurons respectively when replicates were considered. The standard deviation was higher when all experiments were considered, 58.1 ± 8.2 % and 43.5 ± 10.0% respectively.

KCl controls had an average of VIP slightly higher than that observed with ENS cultured with T84 alone 0.010 ± 0.001 AU *vs* 0.09 ± 0.001 AU and 0.010 ± 0.003 AU vs 0.009 ± 0.002 AU when all experiments were considered. Concerning the comparison of the 34 experiments or the duplicates inside each experiment, standard deviations of negative controls were almost identical to those of ENS exposed to KCl.

In conclusion these analyses demonstrate validated our co-culture model in terms of repeatability and reproducibility.

Assessment of neuronal survival in presence of bacterial strains

Next, we determined whether bacterial strains could modify neuronal survival in the co-culture model. Neuronal survival was assessed by evaluating in each well and for each bacterial strain, either the number of total neurons (Hu-IR) per ganglion (Figure 1A) or the extracellular NSE in the culture medium (Figure 1B) as function of bacterial strains. Ganglia contained an average of 19.9 ± 1.7 neurons ranging from 16.3 ± 0.9 (*Bifidobacterium longum*) to 25.0 ± 2.5 neurons (*Bifidobacterium bifidum*), suggesting that bacteria had no impact upon the survival of neurons. NSE release ratio were on average 0.044 ± 0.030 and ranged between 0.011 ± 0.018 (*Bifidobacterium breve*) and 0.166 ± 0.153 (*B. longum*).

Bacterial effect upon the cholinergic phenotype

Next, we measured the effects of the 69 individual strains on the cholinergic phenotype and data are shown in Figure 2 and in Table 2.

We further analysed the modulation of the cholinergic phenotype with the objective to identify whether there was a correlation between phylogenetic clusters and modulatory potential. A variance components analysis (Supplemental data Table 2) showed that the effect of genus accounted for 25% of the total variance, while the species parameter contributed only for 9% of total variance. The strain effect was the component that most contributed to the variance with 66% of total variance (Supplemental data Table 2) demonstrating that this bacterial phenotype occured in different species and is highly strain-specific.

Two groups of strains that reduced or increased ChAT-IR neurons proportions were identified. Thirteen strains that belong exclusively to the *Bifidobacterium* group, specifically to the *Bifidobacterium longum* and *Bifidobacterium bifidum* species, were able to significantly reduce the proportion of ChAT-IR neurons (from 2 to 5 fold) as compared to the mean of all strains (Table 2). One additional *Bifidobacterium breve* CNCM Bbr19 strain still had the tendency to reduce the proportion of ChAT-IR neurons (p=0.07). Interestingly, most of the strains that had the ability to significantly increase the proportion of cholinergic neurons as compared to the mean of all strains belonged to the *Lactobacillus* genus (Table 2). Seven strains of the *Lactobacillus* genus were identified in this respect: 3 *Lactobacillus rhamnosus* (Lr61, CNCM I-4317, Lr49), 1 *Lactobacillus bulgaricus* CNCM I-1632 and one *Lactobacillus helveticus* (Lh118) and two strains of the *Bifidobacterium* genus: one *Bifidobacterium breve* Bbr31 and one *Bifidobacterium bifidum* CNCM I-4320 strains. Seven other *Bifidobacteria* as well as one *Lactobacillus* strain (*L. bulgaricus*) tended also to increase the proportion of cholinergic neurones as compared to mean of all strains tested.

Bacterial effects on intracellular VIP

In this part, we measured the effects of the 69 individual strains on neuronal VIP content and data are shown in Figure 3.

We further analysed the modulation of the VIPergic phenotype with the objective to identify whether there is a correlation between phylogenetic clusters and modulatory potential (supplemental data). Strain and species contributed respectively with 58.4% and 41.6% of the total variance. Genus did not account to total variance of VIP.

As shown in table 2 we first observed that VIP was less prone to bacterial modulation than ChAT was. Indeed among all 69 strains, only 6 strains were able to significantly modulate VIP level, as compared to 22 strains able to modulate ChAT. Among the *Bifidobacterium genus*, three strains (*Bifidobacterium bifidum Bb79, Bifidobacterium bifidum Bb80* and one *Bifidobacterium animalis CNCM I-4602)* and one strain of *Lactobacillus plantarum CNCM I-4318* were able to significantly increase the amount of VIP as compared to the mean of all strains tested. Two additional *Bifidobacterium bifidum* strains (Bb81, CNCM I-4320) tended to increase VIP amounts (p values between 0.05 and 0.1).

One strain of the *Lactobacillus* genus reduced significantly VIP expression in the ENS (*Lactobacillus helveticus Lh118*) as well as one *Bifidobacterium* strain (*Bifidobacterium breve Bbr27)*. Finally, two additional strains, one of *Bifidobacterium longum* Bll94 and one of *Lactobacillus bulgaricus Ldb595* tended to reduce VIP expression in the ENS (p values between 0.05 and 0.1).

Bacterial effect on both cholinergic and VIPergic phenotypes.

The strains capacity to modulate both ChAT and VIP were rare. Indeed among all the 69 strains, only two strains could be identified with this property (Figure 4). One strain *Lactobacillus helveticus Lh118* increased ChAT and reduced VIP and one strain *Bifidobacterium bifidum* had the opposite effect. In addition, one *Bifidobacterium bifidum Bb80* strain significantly decreased ChAT and had the tendency to increase VIP.

TER assay

We also evaluated whether Bifidobacteria and Lactobacilli strains were able to modulate the resistance of monolayers of the T84 cells after 4h of infection with bacteria. TER values measured at 4h were normalized to their baseline value prior to co-incubation (T0). We next aimed to analyze the involvement of the genus, species and strains that accounts for the modulation of TER using a variance components analysis (Table 3). Variance due to strain accounted for 71.5% of the total variance, species accounted for 20.7% and genus for 7.7% of total variance.

Among all the 69 strains, only three Bifidobacteria strains were able to significantly increase the epithelial resistance as represented by the ratio of TER T4h/T0h (Figure 5 &). These three strains belonged to *Bifidobacterium bifidum* and *Bifidobacterium longum* (Bll96) species, one *B. breve* (Bbr27) and two *L. rhamnosus* (Lr46 and Lr49) with the

Bifidobacterium bifidum CNCM I-4319 allowing the strongest increase of epithelial barrier resistance. Interestingly one *Bifidobacterium bifidum Bb80*, previously identified as a strain able to increase VIP expression and reduce ChAT expression, had also the capacity to increase TER. These results suggested that this strain could maintain GI barrier by acting on several cellular mechanisms.

Eight strains of the bifidobacteria group were able to significantly reduce T4h/T0h TER ratios (six *Bifidobacterium longum* and two *Bifidobacterium bifidum* strains).

Discussion

This study has described and validated a tool for the exploration of interactions between bacteria, ENS and GI functions. Using this integrative co-culture model, we aimed at determining the ability of Bifidobacteria and Lactobacilli strains to regulate the expression of key neuromediators/enzyme of the ENS. We showed that among 69 strains tested the majority of them (65%) did not modulate ChAT nor VIP expression in primary cultures of ENS. However, we identified a subset of strains of Bifidobacteria and Lactobacilli that were able to differentially regulate, i.e.decrease or increase, the expression of VIP or ChAT in primary cultures of ENS. Interestingly, bacteria-induced neuroplastic changes mainly occurred in a strain-dependent manner whereas genus or species effects were not consistent.

The co-culture model that was used to analyze putative interactions between bacteria and the ENS was composed of primary culture of ENS and intestinal epithelial cell monolayer on Transwell® filters. Such an *in vitro* approach has the advantage to model, in part, the physiological interactions occurring between bacteria and the ENS *in vivo* and could be a tool to screen *in vitro* interactions before *in vivo* experiments. In addition, it can be used to study the direct effects of bacteria upon the intestinal epithelial cells and ultimately upon the ENS. However, conversely, the effects of constituents known 1) to be targeted by bacteria and 2) to modulate ENS phenotype, such as immune cells, fibroblasts or endothelial cells are not present in our model. This is therefore a limitation of this *in vitro* approach.

This model has been previously developed in our laboratory to demonstrate that long-term co-culture (i.e. 3 days) of ENS with intestinal epithelial cells induced neuroplastic changes in enteric neurons (Moriez et al., 2009). In the current study, we validated the reproducibility and repeatability of the model in order to assess the impact of bacteria upon the expression of ChAT and VIP. In parallel, butyrate was used to evaluate the response of cholinergic neurons as previous studies have demonstrated its ability to increase ChAT expression both *in vitro* and *in vivo* (Soret et al., 2010; Suply et al., 2012). Additionnaly, KCl was used as to evaluate the response of VIPergic neurons as a previous study had shown that KCl increased expression of VIP in the primary culture model of ENS used (Chevalier et al., 2008).

A major finding of the study was the demonstration that some strains of Bifidobacteria and Lactobacilli were able to modulate the neurochemical phenotype of the ENS. These neuroplastic changes were observed following 8h of co-culture of the ENS with T84 infected cells with various bacterial strains and maintained an additional 22h without epithelial cells.

T84 monolayers were maintained in contact with bacteria for 8h. This duration was sufficient to induce changes both in IEB and ENS. This is consistent with previous studies showing that bacteria such as *S. flexneri* or *C.difficile* were able to modify neuromediators expression in the ENS (Coron et al., 2009; Castaglioulo et al.,1998) within a few hours. In addition, changes in expression of ChAT and VIP by butyrate and neuronal activity (KCl) occurred also within 24h (Chevalier et al., 2008; Soret et al., 2010). Neuroplastic changes induced by bacteria were not associated with changes in survival as the number of neurons per ganglion. In addition, the amount of extracellular NSE released was not significantly modified by strains.

Our study showed that the majority of Bifidobacteria and Lactobacillii strains did not modulate the expression of ChAT or VIP. Only a third of Bifidobacteria and a quarter of Lactobacilli tested significantly modulated ChAT expression. More precisely, Lactobacillii strains only decreased ChAT expression while Bifidobacteria strains mainly increased ChAT expression. In contrast, a smaller proportion of Lactobacillus (10%) and Bifidobacteria (10%) strains were able to modulate VIP expression. Interestingly, this modulation of the phenotype appeared to be specific of the strains, i.e. only one *Bifidobacterium bifidum* BB80 strain and one *Lactobacillus helveticus* Lh118 strain were able to modulate both VIP and ChAT expression

The reason behind the lower ability of bacteria to modulate VIPergic neurones as compared to cholinergic ones is currently unknown. It could be due in part to our model, as it had a lower sensitivity and reproductibility to study VIPergic responses than cholinergic ones. Alternatively, regulation of VIP phenotype could need a "stronger" stimulus than the one resulting from the interaction between bacteria and monolayer. Indeed, changes in VIP in enteric neurons have mainly been reported during inflammatory conditions such as those induced following pathogens aggressions (*C rodentium*, Conlin et al., 2009; *S flexneri*, Coron et al., 2008) or chemical stimulation (KCl treatment). Similarly, 24h treatment of primary culture of ENS with KCl (40mM) was also able to increase VIP in the ENS (Chevalier et al., 2008).

The factors and mechanisms responsible for individual strain effects upon ENS remain currently unknown and were not explored in this study. However this model provides a powerful tool for future screening aiming at study molecular interactions between bacteria and gut components. As suggested in this study, factors or mechanisms implied seem to be influenced by genus and mainly by the strain.

Bacteria – ENS interactions might proceed by two distinct mechanisms: either via the direct interactions of bacterial metabolites or products and the ENS or via a bacterial

modulation the intestinal epithelial cell phenotype or both. Concerning the first point, bacterial fermentation of resistant starch and associated production of short chain fatty acids such as butyrate was shown to be involved in the induction of neuroplastic changes in the ENS (Soret et al., 2010). In parallel, the ENS has recently been shown to express PRRs that could mediate in part bacteria – ENS communication (Barajon et al., 2009). Indeed, in $TLR2^{-/-}$ mice, neuronal nitric oxide synthase positive neurons and acetylcholine esterase−stained fibers were significantly decreased compared with WT mice (Brun et al., 2013). Interestingly, in TLR4-/- mice, a reduction in the proportion of nNOS enteric neurons was also observed (Anitha et al., 2012). Whether activation of TLR or NOD receptors by bacterial wall or DNA fragments can induce neuroplastic changes observed in our study remains to be demonstrated. Finally, differential modification of intestinal epithelial cells phenotype by bacterial strains could also result in neuroplastic changes as paracrine communication between IEC and ENS has been shown to be involved in the regulation of enteric neuromediators such as VIP (Moriez et al., 2009). Differential modulation of expression of neuromediators by bacteria could be of major interest both from a preventive or therapeutic point of view. Modulation of cholinergic and VIPergic phenotype might directly impact upon two key functions i.e. motility and IEB function, in particular permeability. Concerning motility, increasing acetylcholine and VIP expression in enteric neurons might result in enhanced motility. Indeed, VIP-/- mice exhibit impaired transit time (Lelievre et al., 2007) and VIP has also been shown to regulate expression of Ca (V)1.2 channels on smooth muscle cells thereby enhancing excitation contraction (Shi et al., 2007). Increasing ChAT expression and thereby neuronal acetylcholine expression has also been shown to enhance GI motility (Soret et al., 2010; Suply et al., 2012). Finally, in pathologies associated by reduced motility such as slow transit constipation reduced expression of ChAT and VIP have been reported (King et al., 2010). Alternatively, reducing ChAT and VIP expression might lead to reduced motility and be of interest in pathologies with enhanced motility such as IBS. Concerning, modulation of barrier functions, increasing VIP expression might be of interest to reduce permeability. Conversely, reducing ChAT expression might reduce paracellular permeability as acetylcholine has been shown to increase permeability (Hallgren et al., 1999). Interestingly, in animal models of IBS, increased acetylcholine content has been observed in the colon and was associated with increased paracellular permeability (Gareau et al., 2007). Therefore, strains reducing ChAT and enhancing VIP expression such as *B. Bifidum* Bb80 or *B. breve* Bbr31might be of particular interest to reinforce intestinal epithelial barrier.

In conclusion, our study demonstrated the ability of bacterial strains to induce neuroplastic changes in the ENS in a strain dependent manner. Although mechanisms and mediators involved remain elusive, this approach could serve as a basis for further investigations. Our results, once validated *in vivo*, could open the door for developing bacteria-based approaches to selectively regulate neuromediators expression and ultimately gut functions depending on the therapeutical applications requested, in particular for motility or IEB dysfunctions as seen in IBD or IBS.

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Tables

	% ChAT-IR neurons		Intracellular VIP (arbitrary units)	
	Positive controls (sodium butyrate)	Negative controls (T84 alone)	Positive controls (KCl)	Negative controls (T84 alone)
Mean of experiments	58.1	43.5	0.010	0.009
SD within all runs (n=34)	8.2	10.0	0.003	0.002
SD within replicates in run	4.0	3.1	0.001	0.001

Table 1: Regulation of cholinergic and VIPergic phenotype of enteric primary cultures in control conditions. SD: standard deviation.

		Lactobacillus (20 strains)		Bifidobacterium (49 strains)	
	p value	<0.05	<0.1	<0.05	<0.1
Cholinergic phenotype	up	35% (7)	40% (8)	4% (2)	18% (9)
	down	0% (0)	0% (0)	27% (13)	29% (14)
VIPergic phenotype	up	5% (1)	5% (1)	6% (3)	10% (5)
	down	5% (1)	10% (2)	2% (1)	4% (2)

Table 2: proportion of strains with a significant ($p<0.05$) or trend ($p<0.1$) effect on the cholinergic and VIPergic phenotype after co-culture.

	Variance component	% of total
Genus	47.0	7.7
Species	126.3	20.7
Strains	435.5	71.5
Total	608.8	100.0

Table 3: Effects of genus, species and strains on the variability of TER after co-culture

Figures

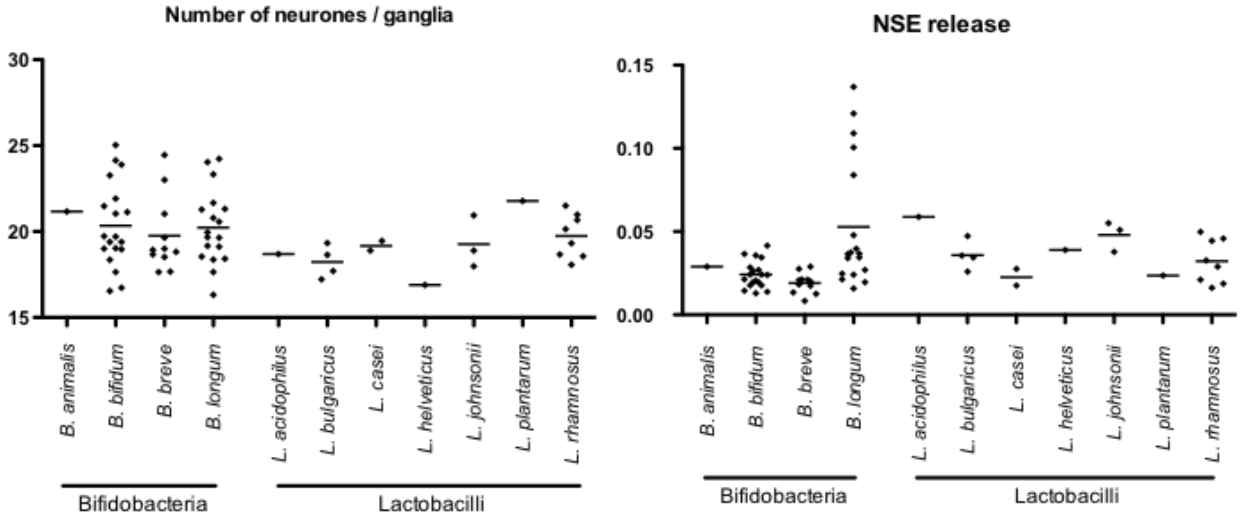

Figure 1: Effect of the bacterial strains added as co-culture on the number of neurones per ganglion (A) and on the NSE release by ENS cells (B). Strains are plotted in species-groups and averages are represented by a horizontal line.

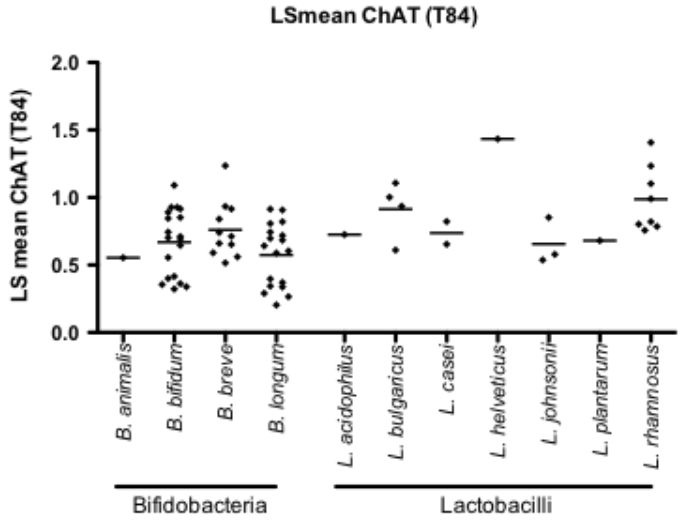

Figure 2: Effect of bacteria on the cholinergic phenotype. Data are expressed as the ratios % ChAT/Hu (strain) / % ChAT/Hu (T84).

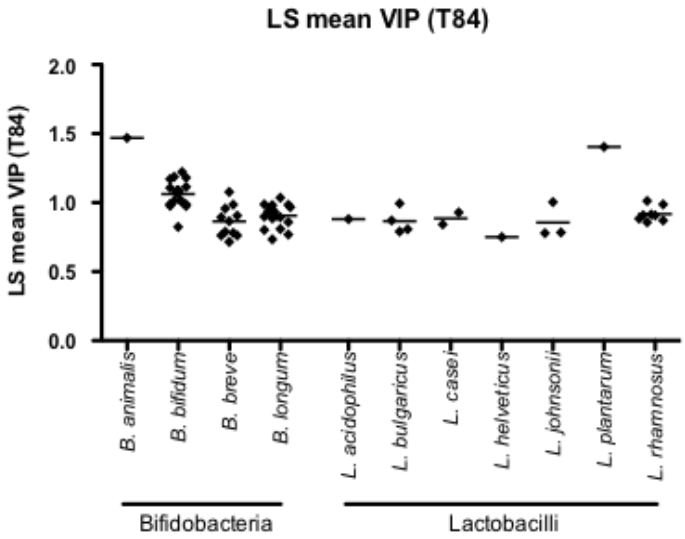

Figure 3: Effect of bacteria on intracellular VIP. Data are expressed as the ratio $VIPi/NSE_{i\,(strain)}$/ VIPi/NSEi(T84).

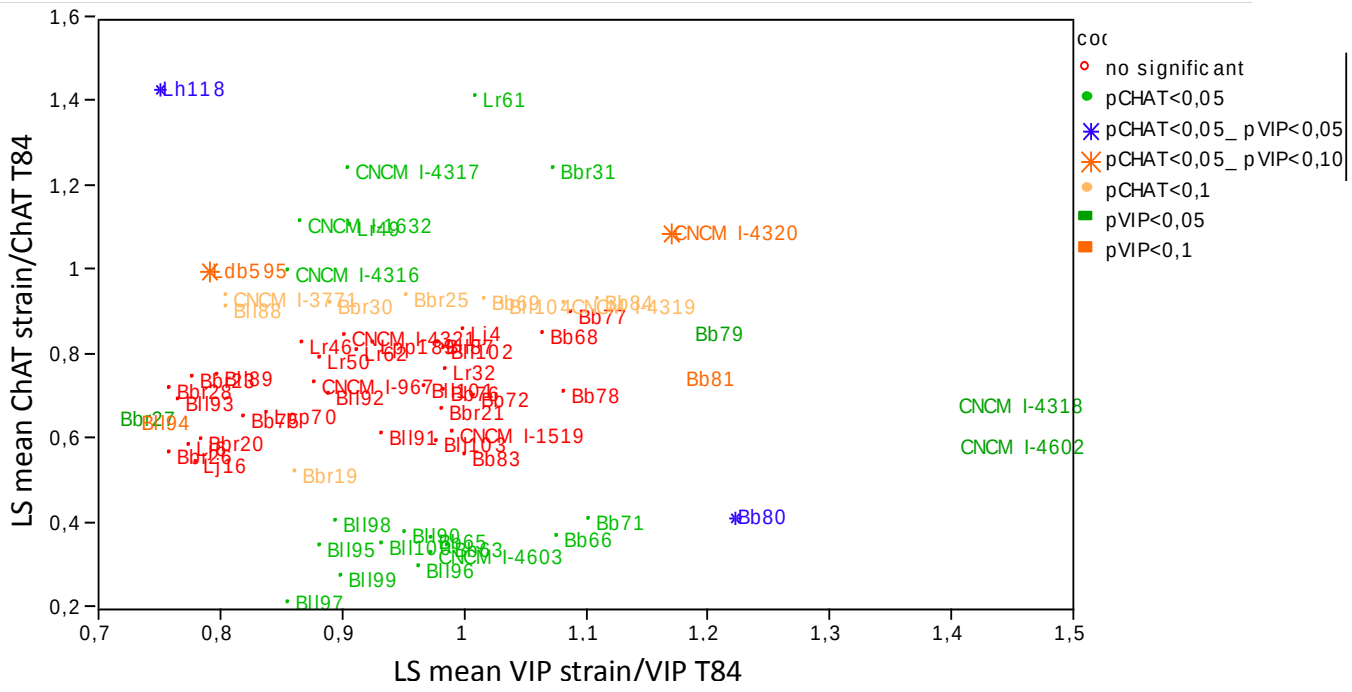

Figure 4: Correlation between cholinergic phenotype and intracellular VIP assessed by p value level. (Bivariate Fit of LS means ChAT normT84 By LS Means VIP normT84).

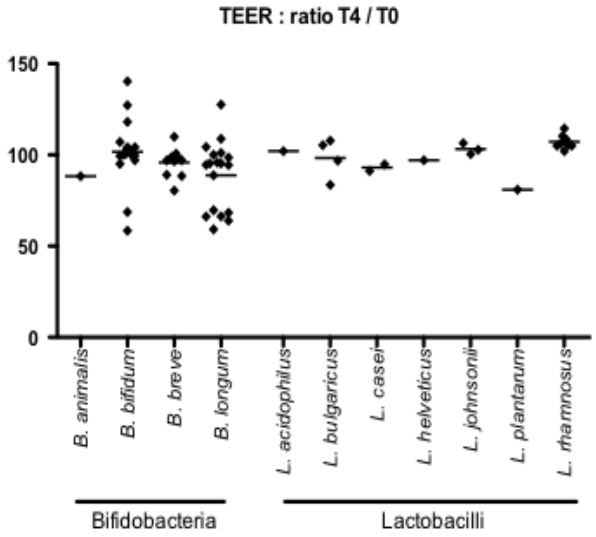

Figure 5: Effect of bacteria on TER expressed as the ratio T4h/T0h.

Supplemental data

Bifidobacteria	
Species	**Strains**
B. animalis	CNCM I-4602
B. bifidum	CNCM I-4319
	CNCM I-4320
	CNCM I-4603
	Bb63
	Bb65
	Bb66
	Bb68
	Bb69
	Bb71
	Bb72
	Bb75
	Bb76
	Bb77
	Bb78
	Bb79
	Bb80
	Bb81
	Bb83
	Bb84
B. breve	Bbr20
	Bbr23
	Bbr25
	Bbr30
	Bbr31
B. breve	CNCM I-4321
	Bbr19
	Bbr21
	Bbr26
	Bbr27
	Bbr28
B. longum	BII87
	BII88
	BII91
	BII92
	BII93
	BII94
B. longum	BII89
	BII90
	BII95
	BII96
	BII97
	BII98
	BII99
	BII100
	BII101
	BII102
	BII103
	BII104

Lactobacilli	
Species	**Strains**
L. acidophilus	CNCM I-967
L. bulgaricus	CNCM I-1519
	CNCM I-1632
	CNCM I-3771
	Ldb595
L. casei	Lpp70
	Lpp189
L. helveticus	Lh118
L. johnsonii	Lj4
	Lj6
	Lj16
*L. plantarum**	CNCM I-4318
L. rhamnosus	CNCM I-4316
	CNCM I-4317
	Lr32
	Lr46
	Lr49
	Lr50
	Lr61
	Lr62

Table 1: Species and strains used in this study.

*cultured in TGYH medium during 24h (initially identified as a *Bifidobacterium* strain)

	Variance component		% of total variance	
	%ChAT-IR neurons	Intracellular VIP	% ChAT-IR neurons	Intracellular VIP
Genus	0.021	<0.001	25.0	0.0
Species	0.008	0.019	9.2	41.6
Strains	0.054	0.026	65.8	58.4
Total	0.083	0.045	100.0	100.0

Table 2: Effects of genus, species and strains on the variability of cholinergic and VIPergic phenotype after co-culture

3- Impact de souches bactériennes sur la maturation du système nerveux entérique et des fonctions digestives

Titre : *In vivo* modulation of gastro-intestinal functions and enteric nervous system by selected strains of Lactobacilli and Bifidobacteria.

Auteurs : Etienne Suply, Margarida Ribeiro Neunlist, Philippe Aubert, Julien Chevalier, Mandy Biraud, Julie Jaulin, Raphaël Moriez Pascale Rondeau, Biliana Lesic, Michel Neunlist

Objectifs : tester l'effet de bactéries selectionnées sur la mise en place des fonctions digestives du raton (motricité et permeabilité) ainsi que sur la maturation du phénotype neuro-glial du SNE.

Implication personnelle : j'ai réalisé l'ensemble des expériences, l'analyse des résultats et la rédaction de la publication en collaboration avec Margarida Ribeiro Neunlist.

Abstract

Background: To improve gut health, the use of probiotics, benefic bacterial strains, may impact on several gut components (epithelial cells, immune cells) or symptoms (constipation, visceral sensitivity). However, bacterial induced changes in the enteric nervous system (ENS), a key regulator of GI function and homeostasis is poorly studied. Thus, this study aimed to assess in newborn rats the modulation of GI functions and ENS phenotype by a set of putative probiotic strains previously selected.

Method: We studied a set of 3 lactobacilli (*bulgaricus, helveticus, plantarum*) and 3 bifidobacteria (*animalis, animalis lactis, bifidum*) selected *in vitro* in previous experiments. Rat pups were daily fed by cultured strains between postnatal day (PND) 7 and PND 17. Gut motility was assessed *in vivo* (PND 20-21, whole gut transit time, colonic transit time) and *ex vivo* (PND 22, organ chambers, proximal and distal colon). Paracellular permeability (sulfonic acid flux) was also measured *in vivo* (PND 21) and *ex vivo* (PND 22, Ussing chambers, proximal and distal colon). ENS phenotype of the colon was evaluated by assessment of neuromediators (Acetylcholine, VIP, serotonin) and glial markers (GFAP, S100β, Sox10). Colon inflammation status was also studied (Il-10, IFNγ, Il-1β).

Results: We showed in this study a high diversity of response of the colon after treatment by bacterial strains. Strains were able to either increase either decrease some parameters of motility and permeability *in vivo* or *ex vivo*. Regarding the neuronal phenotype, few neurochemical modulations were observed while intense modifications of glial markers were observed. Inflammatory status of the colon was poorly modified.

Conclusion: We observed a highly diversity of effects of bacterial strains on GI functions and ENS phenotype. This approach could serve as a basis for further exploration and validation of putative benefic bacterial strains upon gut health.

Introduction

Interplay between microbiota and gut health is currently undergoing a growing interest. Microbiota is now considered to be a major actor of gut functions and is composed by up to 10^{14} bacteria from 7 phyla, mainly firmicutes and bacteroidetes (Arumugam *et al.* 2011). Although similarities in microbiota composition are observed among the individuals, it seems that gut flora of each is different, leading to the concept of microbiota fingerprint. This complexity may be the result of several factors (mode of delivery, feeding practices, antibiotic use, pathogen exposure, ...) acting from its establishment to a stable state reached in childhood (Arrieta *et al.* 2014).

Interestingly, specific gut composition profiles were observed in gastro-intestinal (GI) diseases. As example, in necrotizing enterocolitis, it was shown a higher proportion of proteobacteria associated with a decrease of in firmicutes in feces samples compared to healthy control newborns (Berrington *et al.* 2014). In adults, particularly in Crohn's disease, the lower rate of *Faecalobacterium prausnitzii* in colonic flora is still controversial (Kang *et al.* 2010). However, this bacterial strain is able to produce butyrate, a short chain fatty acid, from digestion of dietatry fibers. This metabolite may be a mediator of putative effects of flora on gut functions as it was shown to induce a prokinetic effect on the colon by a modulation of the enteric nervous system (ENS) phenotype (Soret *et al.* 2010).

The ENS is a network of neural crest derived cells which has colonized the GI track during embryonic development (Le Douarin and Teillet 1973). Two layers are mainly described with interconnections: the submucosal plexus (under the submucosal layer) and the myenteric plexus (within the muscle layer). By releasing neuromediators, these plexus are major regulators of GI functions: barrier permeability and motility respectively (Schemann and Neunlist 2004). Regarding motility, muscle contractions are induced by acetylcholine or substance P and relaxation by NO or ATP. Regarding intestinal permeability, VIP reinforces epithelial barrier, decreasing intestinal permeability (Schemann and Neunlist 2004). Interestingly, the ENS phenotype shows maturation patterns after birth, a key period of microbiota establishment. In particular, the proportion of cholinergic neurons and nitrergic neurons of the colon myenteric plexus increases especially during rat weaning (de Vries *et al.* 2010). Reinforcing this message, in germ-free animals, ENS phenotype was shown to be modified as compared to controls (fewer neurons in myenteric ganglia, higher proportion of nitrergic neurons) as well as small intestine motility (decreased in frequency and amplitude) (Collins *et al.* 2014). Moreover, neuron excitability was shown to be decreased in germ free

animal, partially recovered after microbial colonization (McVey Neufeld *et al.* 2013). However, the impact of bacterial strains on the ENS phenotype in conventionnal animals is poorly studied, in particular concerning key neuromediators involved in the control of GI functions.

In this context, this study aimed to evaluate putative modulation of GI functions and ENS phenotype induced by orally dispensed bacteria in specific pathogen free rats.

Method

Selection of bacterial strains

We used an *in vitro* model of co-culture previously described by Ribeiro *et al*. Briefly, rat primary cultures of ENS composed by neurons and EGC were co-cultured with a monolayer of epithelial cells (T84) seeded on a transwell filter® (Corning, NY, USA). Bacterial strains (lactobacilli or bifidobacteria) were dropped in the apical medium and after co-culture, ENS phenotype was studied. We selected 6 strains according to their ability to induce modulation of cholinergic and/or ViPergic phenotype on this co-culture model.

Strains that increased the expression of ChAT were *Lactobacillus bulgaricus* (100130, CNCM I-1632), *Lactobacillus helveticus* (112003, LH 118) and *Lactobacillus plantarum* (121304, CNCM I-4318). *Bifdobacterium bifidum* (154064, BB 83) decreased the expression of ChAT. Strains that increased the expression of VIP were *Lactobacillus plantarum* (121304, CNCM I-4318) and *Bifidobacterium animalis* (153004, CNCM I-4602). *Bifidobacterium animalis lactis* (173010) had no effect on the expression of ChAT or VIP.

Animal model

ANIMALS

Animal experiments were conducted in the Unité de thérapeutique expérimentale of the Institut de recherche thérapeutique of the University of Nantes. All procedures were approved by the ethics committee in animal experimentation of the Loire-Atlantique department. Primiparous pregnant Sprague-Dawleys rats (Janvier, Le Genest, France) were delivered at 13-14 days of gestation, placed in individual cages with free access to food and water. After birth and throughout the whole duration of the experiment, pups were left in the presence of their mother. All pups of each scope were treated with the same substance to avoid any contamination within the litter. Pups were identified by a numbered ring on the ear. Pups were euthanized by cervical dislocation at 22-23 postnatal day (PND) 22-23. The colon was removed immediately by laparotomy and kept in a 4°C Krebs solution containing (in mM) 117.0 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 $NAH_2 PO_4$, 25.0 NaHCO3, 2.5 $CaCl_2$ and 11.0 glucose).

EXPERIMENTAL GROUPS

We performed seven experimental conditions depending on the substance administered: *Lactobacillus bulgaricus* (100130), *Lactobacillus helveticus* (112003), *Bifidobacterium animalis* (153004), *Lactobacillus plantarum* (121304), *Bifidobacterium animalis lactis* (173010), *Bifdobacterium bifidum* (154064) and Control (phosphate buffer saline, PBS). Scopes were randomized to test each experimental condition (10 rats per litter). Strains were tested twice each independently.

BACTERIAL STRAINS ADMINISTRATION

Bacterial strains were daily administered between PND 7 and 17 (between 9:00 and 10:00 am). To avoid acute stress due to intra-gastric feeding, 100 µL of the solution containing PBS or PBS+strains (10^8 UFC/100µL) were slowly dropped in the mouth of each pup at the rate of swallowing. Animals handled carefully avoiding any regurgitation. This amount of bacteria was determined in preliminary experiments as the maximal amount livable for rat pups. In these preliminary experiments, while 10^7 to 10^8 UFC were well tolerated following tolerance criteria defined below, 10^9 UFC induced mortality rate around 40%.

TOLERANCE OF ADMINISTRATION

Before each administration of the solution containing bacterial strains, clinical tolerance was evaluated following these criteria: weight gain, general comportment, motor activity development, skin aspect, and abdominal inspection. These criteria were validated by the committee in animal experimentation directed by a veterinarian.

Bacterial strains preparation

Bacterial strains were obtained by collaboration with Danone research (Palaiseau, France). Lactobacilli were cultured in Man, Rogosa and Sharpe medium (MRS) (Biokar Diagnostics, Beauvais, France) at 37°C under air atmosphere conditions and bifidobacteria with tryptone peptone (Becton Dickinson-USA) basis supplemented with glucose (Sigma, France), yeast extract (Becton Dickinson-USA) and hemine (TGYH medium, Calbiochem, France) at 37°C under anaerobic conditions (AnaeroGen™, Oxoid, England). The stock solution was constituted with 1 mL of the last culture added with 5,6% of DMSO and frozen(-

80°C). The pureness of each culture was verified by optical microscopy (x100) with methylen blue (Réactifs Ral, Martillac, France) stained slides as well as plate spreads.

In preliminary experiments we determined the procedure to obtain the correct amount of bacterial strains per rat (100µL, 10^9 CFU/ml) and the time to obtain strains at the end of their exponential growth phase. Thus, we first performed a pre-culture of 24 hours in adapted conditions. Then we inoculated a flask of fresh culture medium (37°C) at several concentrations and measured the optical density (BioSpec-mini, Avantec, France, wavelength of 580 nm) of the culture medium and pH at different time-points. To determine the correspondence between the optical density observed and the number of CFU in the culture medium, counts on plates (agar culture medium adapted to strain) were performed in duplicate by seeding the last three dilutions of a series of six serial dilutions (peptone water). The number of CFU per dish was determined by visual reading after 48-72 hours of culture at 37°C under anaerobic conditions. The plateau phase was determined by graphical analyses showing the optical density and the pH of the culture over the time. We finally choose the best compromise between the initial inoculate and the time of culture.

48 hours before the first gavage, 100 µL of stock solution were seeded in 10 mL of appropriate medium. After 24 hours of culture, the bacteria were cultured according to the protocol previously established. After the culture time required (16 to 22 hours depending on the strain), the optical density of the culture medium were measured. The required volume of medium to obtain 10^8 CFU/100µL was calculated and then collected and centrifuged (4000 rpm, 15 min, 20°C). The bacterial pellet was then washed once in 10 ml of 1X PBS and then centrifuged again (4000 rpm, 15 min, 20° C) before being suspended in 1X PBS (100µL per rat). The pureness of cultures was regularly checked by microscopic observation and also by observation of cultured dishes for bacterial counting. To ensure the pureness of cultures, a new tube of stock solution was thawed every 3 days. At least one count was conducted during 10 days of feeding.

Evaluation of gut functions

EVALUATION OF MOTILITY

In vivo

Oro-anal transit: Whole gut transit time

This experiment was performed at PND 21 in parallel with the faecal pellet output counting and the measurement of the intestinal permeability. To avoid the influence of the circadian cycle, all the experiments were started at 9:00 am. Rats were fed (intra-gastric feeding) with a solution of carboxy methyl cellulose 0.5% containing carmine red dye (200µL, 60mg/mL). Rats were isolated in individual cages without access to either food or water until the emission of the first stool stained. The time required to observe the first stool stained in red has been considered as an index of the whole gut transit time.

Colonic transit: Faecal pellet output (FPO)

Rats were isolated in individual cages at PND 21 without access to either food or water at 2:30 pm. Faeces were collected as soon as issued during 2 hours. The water content of faeces was determined by subtracting the wet weight by the dry weight of faeces (drying 7 days, 50°C) and expressed as percentage.

Distal colonic transit: Bead latency

The distal colonic transit time was assessed at PND 20. To avoid the influence of the circadian cycle, all the experiments were performed at 9:30 am. A 2 mm-diameter glass bead (Sigma) was inserted 5 mm in the colon using a 5 French gauge diameter polished-end ureteral catheter (Porgès, Le Plessis Robinson, France). After bead insertion, pups were isolated in individual cages without access to either food or water. Distal colonic transit time (bead latency) was determined by measuring the time required for the expulsion of the bead.

Ex vivo

After sacrifice at PND 22 by cervical dislocation, colons were removed from the abdominal cavity and placed in cold oxygenated (5% CO_2, 95% O_2) Krebs solution. Segments of proximal and 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 minutes. Isometric contractions were recorded with force transducers (Radnoti, Italy) and data acquired onto a computer equipped with the Labchart V7.2 software (ADI, Bremen, Germany). Activation of enteric neurons was performed by electrical field stimulation (EFS) using a stimulator connected to two platinum ring electrodes (10 Volts, duration of pulse train: 10 seconds, pulse duration: 300 microseconds; frequency: 30Hz). The area under the curve during the stimulation was calculated. This procedure was repeated 3 times with 10-minutes periods between stimulations. Data were normalized to the weight of the tissue.

EVALUATION OF INTESTINAL PERMEABILITY

In vivo

This experiment was performed at PND 21 in parallel with the faecal pellet output counting and the measurement of the whole gut transit time. To avoid the influence of the circadian cycle, all the experiments were started at 9:00 am. Rats were fed (intra-gastric feeding) with a solution of carboxy methyl cellulose 0.5% containing sulfonic acid (200 µL, 578 Dalton, 10mg/mL) and red carmine (60mg/mL). Rats were isolated in individual cage without access either to food or water. A blood sample was collected in the facial vein 30 and 60 minutes after intra-gastric feeding and preserved at 4°C in a tube containing 5µL of PBS-EDTA (0.25M). Plasmas were removed after centrifugation (3 000 rpm, 20 min, 4°C). After dilution with PBS (1/30), the optical density was determined (duplicate) (Varioskan, Thermo Fisher Scientific, Courtaboeuf, France) as a reflect of the paracellular permeability.

Ex vivo

After sacrifice at PND 22, colons were removed from the abdominal cavity and placed in cold oxygenated Krebs solution. Full-thickness segments of distal colon were mounted in 2-mm-diameter Ussing chambers (Easy mount, Warner Instrument, Hamden, CT). Tissues were maintained at 37°C in DMEM (Invitrogen, Cergy Pontoise, France) containing 0.1% fetal calf serum (AbCys s.a., Paris, France) continuously oxygenated. After 30 minutes of equilibration, 200 µl of apical medium was replaced by 200 µl of sulfonic acid solution (578 Dalton, final concentration: 0.1mg/mL) (Invitrogen). The fluorescence level in the basolateral chamber was measured every 30 minutes during 150 minutes using a fluorometer (Varioskan,

Thermo Fisher Scientific, Courtaboeuf, France). The slope of the fluorescence intensity over time was determined by using a linear regression fit.

Biological samples

ACETYLCHOLINE AND VIP ASSAYS

Pellet-free tubular segments of distal colon were placed in RIPA buffer (Millipore, Temecula, CA, USA) and frozen at -80°C. Acetylcholine and VIP concentrations were determined in tissue homogenates (Amplex Red, acetylcholine/acetylcholinesterase assay kit, Invitrogen/ VIP EIA assay kit, Bachem, Bubendorf, Switzerland; respectively) and normalized to the protein amount of the sample (BCA, Thermo Fisher Scientific).

RNA EXTRACTION

Pellet-free tubular segments of distal colon were placed in RP1 buffer (Macherey Nagel, Düren, Germany) with 6-beta-mercapto-ethanol (1%) and frozen at -80°C. RNAs were extracted following the instructions of the kit. After extraction, RNA's levels were assessed with Nanodrop (Thermo Fisher Scientific).

PRIMERS

Primers Sense	T°C	Gene	Lenght	Tm	%GC	Position	Séquence primers 5' --> 3'	Taille amplicon	Séquence Amplicon	Taille du gène sans intron	Taille du gène avec introns	nbr d'introns
Forward	55	VIP	20	59	55	232-251	ggccaccttcttcagtgagt	107	ggccaccttcttc	1501	1501	6
Reverse	55	VIP	20	59	50	319-338	ggcattctgcaagatgtcag	107	ggccaccttcttc	1501	1501	6
Forward	55	ChAT	19	60	58	574-592	gagcctgagcatgtcatcg	71	gagcctgagcat	1938	49020	16
Reverse	55	ChAT	20	60	45	625-644	cggcggaaattaatgacaac	71	gagcctgagcat	1938	49020	16
Forward	60	ZO1	22	59	41	1231-1252	tcaacacatgaagatgggattc	77	tcaacacatgaa	6639	65759	27
Reverse	60	ZO1	20	60	55	1288-1307	cgcaaacccacactatctcc	77	tcaacacatgaa	6639	65759	27
Forward	55	Ocln	21	60	52	1170-1190	tgtataagtcaccgcctctgg	72	tgtataagtcacc	4150	50396	8
Reverse	55	Ocln	20	59	50	1222-12241	tgaagtcatccacggacaag	72	tgtataagtcacc	4150	50396	8
Forward	60	Cldn1	20	59	50	380-399	cgactccttgctgaatctga	103	cgactccttgctg	3219	15156	3
Reverse	60	Cldn1	19	59	53	464-482	gccaatggtggacacaaag	103	cgactccttgctg	3219	15156	3
Forward	60	S100-beta	21	59	48	220-240	aaggagctcatcaacaacgag	87	aaggagctcatc	1484	33069	2
Reverse	60	S100-beta	20	60	55	287-306	gtccagcgtctccatcactt	87	aaggagctcatc	1484	33069	2
Forward	55	Sox10	20	60	50	1248-1267	atgtcagatgggaacccaga	74	atgtcagatggg	3029	11246	4
Reverse	55	Sox10	19	60	58	1303-1321	gtctttggggtggttggag	74	atgtcagatggg	3029	11246	4
Forward	60	Tph1	20	60	45	949-968	aaattggcctggcttctctt	91	aaattggcctgg	1726	21481	11
Reverse	60	Tph1	20	59	50	1020-1039	ttgcacagtccaaactccac	91	aaattggcctgg	1726	21481	11
Forward	60	SerT	20	60	45	921-940	aaaggcgtcaaaacatctgg	74	aaaggcgtcaa	2587	21102	12
Reverse	60	SerT	21	59	52	974-994	agcaggacagagaggacaatg	74	aaaggcgtcaa	2587	21102	12
Forward	55	IL1-beta	20	59	50	149-168	gctgacagaccccaaaagat	74	gctgacagacc	1329	6446	6
Reverse	55	IL1-beta	20	60	55	203-222	agctggatgctctcatctgg	74	gctgacagacc	1329	6446	6
Forward	55 et 60	Rps6	20	59	50	162-181	tgctcttggtgaagagtgga	132	aaggttttcccat	808	2861	4
Reverse	55 et 60	Rps6	21	59	43	273-293	caagaatgccccttactcaaa	132	aaggttttcccat	808	2861	4

STATISTICAL ANALYSIS

Datas obtained after each condition were pooled and expressed as mean ± SD. Expression of mRNA were expressed as fold change of control condition. Statistical analyses were performed using one-way ANOVA followed by post hoc Dunnet test. P-values < 0.05 were considered as statistically significant.

Results

Impact of bacterial supplementation on GI function

GI MOTILITY

Whole gut transit time was performed to evaluate the global motility (figure 1A). This time was significantly decreased (enhanced transit) after treatment with L. *plantarum* and B. *animalis* while it was significantly increased (slower transit) after B. *animalis lactis*. To characterize more specifically the colonic transit, we performed a fecal pellet output counting. After 2 hours, the number of feces collected was similar in all groups. However, the total weight of feces issued (wet and dried feces) was lower after treatment by L. *helveticus* (figure 1B). Finally, we measured the bead latency time to assess the distal colonic transit time. The latency was increased (slower transit) after B. *bifidum* treatment (figure 1C).

Ex vivo evaluation of motility measured the contractile response after stimulation of enteric neurons. This response was strongly decreased after B. *bifidum* in the proximal colon (figure 1D) and increased with L. *bulgaricus* in the distal colon (figure 1E).

GI PERMEABILITY

The overall gut paracellular permeability was assessed measuring *in vivo* the sulfonic acid flux in the blood 1 hour after gavage (figure 2A). The fluorescence of plasma was increased (higher permeability) after L. *helveticus* and B. *animalis lactis*. No strain was able to significantly decrease permeability. The colonic absorption was also evaluated *in vivo* measuring the water content of feces, expressed as percentage of water (figure 2B). It was lower after L. *helveticus*, and higher after L. *bulgaricus*.

Paracellular permeability assessed *ex vivo* in Ussing chamber was increased in the proximal colon after L. *plantarum* treatment (figure 2C). Besides, the sulfonic acid flux throught the distal colon was unchanged whatever the experimental condition (figure 2D).

Modulation of mediators involved in the control of GI functions

Neuromediators of the enteric nervous system (figures 3 and 4)

The acetylcholine amount in the proximal colon was increased after L. *bulgaricus* treatment but mRNA expression levels were unchanged in all conditions. In the distal colon,

acetylcholine amounts were decreased in all experimental groups while mRNA expressions were unchanged.

Regarding VIP in the proximal colon, its expression was increased for L. *helveticus* and B. *animalis lactis* treatment whereas mRNA expression level was increased by B. *animalis* and B. *bifidum*. In the distal colon, VIP concentrations as well as mRNA expression levels were unchanged.

Enteric glial cells markers (figure 5)

GFAP mRNA expression was not changed in both proximal and distal colon. S100β mRNA expression was decreased after L. *plantarum* and B. *animalis* whereas it was increased by B *animalis lactis* (trend in the proximal colon, and significantly in the distal colon). Sox10 mRNA expression was increased after L. *helveticus*,B. *animalis lactis* and B. *bifidum* in both proximal and distal colon.

Serotoninergic phenotype (figure 6)

The serotoninergic pathway was investigated by evaluation of TPH2 and SLC6 mRNA expression. In the proximal colon, TPH2 mRNA expression was increased after L. *bulgaricus*, L. *plantarum* and B. *bifidum* while SLC6 mRNA expression was only increased by L. *bulgaricus*. In the distal colon, TPH2 and SLC6 mRNA expression were increased only after L. *bulgaricus*. In parallel, we assessed TPH1 mRNA expression. It was increased after B. *animalis lactis* in the proximal colon and after L. *bulgaricus* and L. *plantarum* in the distal colon.

Impact of bacterial strains on the expression of junctional proteins (figure 7)

ZO-1 mRNA expression was unchanged in both proximal and distal colon (A, B). B *Bifidum* increased mRNA expression of occludin in the proximal and distal colon as well as B *animalis lactis* in the distal colon (C,D). mRNA expression of Claudin 1 was decreased only in the sistal colon by L plantarum and B animalis (E, F)

Impact of bacterial supplementation on intestinal inflammation (figure 8)

Il-10 mRNA expression was not modified in proximal nor distal colon whatever the experimental group. Il-1β was increased in both proximal and distal colon after B. *animalis lactis* and B. *bifidum* groups. IFN-γ was strongly increased in L. *bulgaricus* condition in the proximal colon and unchanged in the distal colon.

Discussion

Our study is to the best of our knowledge, the first comprehensive study aimed at characterizing the impact of different bacterial strains upon GI functions and the ENS, a key regulator of GI motility and permeability. Overall, our results demonstrate strain dependent effects upon GI functions and ENS phenotype. These results are consistent with studies reporting 1) strain (*L Rhamnosus* vs *L reuteri*) and region (small vs large intestine) dependent effects of bacteria upon motility (Wu *et al.* 2013) and 2) strain dependent effects upon ENS phenotype (our previous *in vitro* study). Our study also reveals large variability in the effects of a same bacterial strain on diverse functions and gene from run to run. Whether this variability reflect their specific effect based on the host genotype (as study was performed on two different littermates) or result from extrinsic factors remains to be determined. Another potential limitation of our study is the fact that it was performed in a newborn animal model in which the maturation of the ENS and GI functions was not complete. Therefore, although of great and direct interest for studies aimed at improving GI maturation during the neonatal period, our results need to be confirmed in the adult or in animal models of diseases/ symptoms of interest. Concerning the putative age dependent effect of bacterial strains, previous studies have shown that regulation of GI functions or ENS phenotype by key nutritional factors and/or bacterial metabolites was similar in the newborn and the adult although one cannot exclude differences (Suply *et al.* 2012). Another interesting finding was that changes in gene expression (amplitude and number of genes modulated) induce by bacteria appeared to be larger in the proximal colon than in the distal one.

Among the pathways studied that seems to be the most modulated by the bacterial strains tested are genes involved in serotonin synthesis and biodisponibility. In particular, we showed the ability of bacterial strains to differentially modulate serotoninergic pathways. TPH-1, TPH-2 and SERT are key enzymes involved in the biosynthesis/action of serotonin. 5-HT biosynthesis depends on tryptophan hydroxylase 1 (TPH1) and on tryptophan hydroxylase 2 (TPH2). TPH1 is present in the enterochromaffin (EC) cells of the mucosal epithelium and in mast cells whereas TPH2 is located in neurons of the central (CNS) and enteric (ENS) nervous systems (Neal, Parry and Bornstein 2009). Uptake of 5-HT into enterocytes and neurons is mediated by serotonin reuptake transporter (SERT), a serotonin-selective transport protein. Inhibition of SERT or its genetic deletion (SERT-KO) potentiates mucosal and neural actions of 5-HT (Chen *et al.* 2001). Increasing evidences have demonstrated their key role in the control of gut functions. In particular, TPH-2 (-/-) mice show reduced colonic transit (Li *et*

al. 2011). In contrast, deletion of TPH-1 does not affect motility but reduces DSS-induced inflammation. Further supporting a role of 5-HT in intestinal inflammation is data showing that SERT (-/-) mice develop more severe intestinal inflammation (Ghia *et al.* 2009). Interestingly, 5-HT pathways are altered in IBS, although results are still conflicting, and are the target of various therapeutical approaches. In particular, mucosal 5-HT is reduced in IBS-C but similar in IBS-D as compared to control (Wang *et al.* 2007). Interestingly, TPH-1 and SERT mRNA are reduced in IBS-C and D (Coates *et al.* 2004). A recent report by Kerckhoffs *et al*. (Kerckhoffs *et al.* 2012) confirmed the observations of Coates (TPH-1 and SERT mRNA expression are reduced in IBS) but an earlier study of same group showed no change in TPH-1 while an increase in SERT in IBS as compared to control (Kerckhoffs *et al.* 2008). Recently, increased colonic 5-HT release was correlated with mucosal mast cell infiltration and with the severity of abdominal pain/discomfort in IBS patients (Cremon *et al.* 2011). Another recent study in pediatric IBS demonstrated that children showing mild increase in rectal mucosal immune cell counts had higher 5-HT content and lower SERT mRNA expression (Faure *et al.* 2010). Interestingly, platelet SERT is also reduced in IBS-D and associated with reduced levels of SERT mRNA and duodenal immune activation (Spiller 2008). Although, the role of 5-HT in control of barrier functions remains largely unknown, recent data suggest that loss of intestinal SERT is a critical factor in fructose-induced impairment of intestinal barrier function and the subsequent development of steatosis (Haub *et al.* 2010). Interestingly, 5-HT released from EC cells is involved in the translocation of FITC-LPS induced by elevated intraluminal pressure via 5-HT-3 receptors (Yamada *et al.* 2003). Altogether and from a therapeutical point of view reducing serotonin levels either by inhibiting its production (reducing TPH-1) or its biodisponibility (increasing SERT) might be of interest to reducing inflammation and reduce pain. Interestingly, in a phase 2 study, an inhibitor of TPH (LX1031) has been shown to relieve symptoms and increasing stool consistency in patients with non-constipating IBS (Brown *et al.* 2011). Conversely increasing TPH-2 might be of interest to enhance motility and also consequently reduce to inflammation, especially in IBS-C. Our results show, for the first time, that bacterial strain could potentially be used to achieve these goals. Interestingly, in our study, strains that increased (or tended to increase) TPH-2 tend to increase transit time and/or increase water content of feces (*L Bulgaricus*, *L plantarum*, *B animalis* or *B bifidus*) while conversely strains that reduced transit (*B animalis*) or reduced percentage of water in feces (*L Helveticus*) tended to decrease TPH-2. Concerning modulation of 5-HT, *L bulgaricus* appears as a particularly interesting strain to reduce 5-HT biodisponibility and synthesis. Although these interpretations are largely

speculative their efficacy should be tested in animal models or IBS patients in which altered 5-HT homeostasis has been reported.

Concerning bacterial modulation of mediators of the ENS, two strains (*B bifidum* and *L helveticus*) were found to increase VIP mRNA expression. Interestingly, increased VIP mRNA expression was associated with increased mRNA expression of tight junctions proteins (ZO-1 and claudin-1 for *B bifidum* and of ZO-1 for *L Helveticus*). No change in tight junctions proteins mRNA expression was reported for any other strains that did not modify VIP mRNA expression. These data are in line with the known role of VIP in modulating expression of tight junctions such as ZO-1 (Neunlist *et al.* 2003; Conlin *et al.* 2009) and also claudin-1 (Baudry *et al*., submitted). No major changes in ChAT expression by any of the strains tested was reported except for *L Plantarum* that tended to decrease its expression. This ability to decrease ChAT expression might be of particular interest in IBS as increased cholinergic pathways have been identified as responsible for increased transcellular as well as paracellular permeability in IBS (Neunlist *et al.* 2013). The inability of strain to modulate more profoundly VIP or ChAT expression is at first glance surprising as strains were selected during the *in vitro* screening phase for their ability to modulate ChAT and VIP expression. These differences could be due to various reasons such as the differences in dose tested (*in vitro vs in vivo*), the presence of microbiota in the rat pup *vs* its absence *in vitro* and also to the fact that although integrative, the *in vitro* model did not include all cellular components and interactions existing *in vivo*.

Another interesting and novel finding of our study was the ability of bacterial strains to modulate differentially the expression of key markers of enteric glial cells (EGC). EGC have recently been identified as key regulators of intestinal homeostasis (De Giorgio *et al.* 2012; Neunlist *et al.* 2013). In particular, EGC regulate neuronal survival and neuroprotection as well as key neuromediators (ChAT and VIP) involved in control of motility. Besides controlling neuronal functions EGC are also directly involved in the maintenance of intestinal barrier integrity. In particular, they regulate paracellular permeability, intestinal epithelial cell proliferation and differentiation and enhances barrier repair (Neunlist *et al.* 2013). Besides their physiological functions, increasing evidences suggest that under inflammatory conditions EGC shift their phenotype and probably also their functions towards a profile reminiscent to the ones of reactive astrocytes in the CNS. Such changes are characterized by increased expression of GFAP and S100β associated with increased production of cytokines and/or neurotrophic factors such as GDNF. The ability of bacterial strains to modulate EGC

phenotype and functions remains largely unexplored although recent studies have shown that bacteria derived LPS was able to induce "reactive astrogliosis" in EGC (Cirillo *et al.* 2011). However, and conversely, other studies also show that reactive enteric astrogliosis is also associated with increased production of protective mediators such as GDNF (von Boyen and Steinkamp 2010). Therefore, while limiting reactive astrogliosis might be tempting, caution has to be taken as it might at the same time also inhibit its protective effects. Besides inflammation, increased expression of GFAP during the post natal period illustrates probably the post natal maturation of the ENS, and in particular of EGC (Cossais *et al*. unpublished data). Concerning modulation of astrocytes by bacteria, interesting studies have shown that gavage of lactobacillus bacteria (*L fermentum* and *L plantarum*) decreased brain levels of GFAP which was correlated with decreased loco-motor activity in growing animals (Ushakova *et al.* 2009). Interestingly, our study also shows that *L reuteri* reduces GFAP and S100β expression in the ENS, although no functional modifications were identified as associated with these changes. Interestingly, *B. animalis lactis* and *L. bulgaricus* strongly increased GFAP expression in the colon and were associated with changes in motility/permeability. This suggests that in our model of post natal development these strains could increase maturation of the EGC and ultimately GI functions.

Regarding the functional impact of strains upon motility, we found that *B animalis* and *B animalis lactis* could differentially modulate *in vivo* transit time, which one strain increasing it and the other inhibiting it. Interestingly, *B animalis* that increased the transit also increased *ex vivo* the amplitude of the neutrally mediated contractile response. *B animalis* increased paracellular permeability *ex vivo* in the proximal colon and tended to increased it in the distal colon, making of *B animalis* the strain the most able to induce functional changes among the ones tested. *B animalis* was able to increase TPH-2 which might be responsible for its effects upon motility and increased permeability. Conversely, *B animalis lactis* reduced TPH-2 expression which might be directly responsible for the reduced transit induced by this strain. Furthremore, *L bulgaricus* increased water content of stool suggestive of increased colonic motility and this effect was associated with increased TPH-2 expression while *L helveticus* that decreased water content (potentially reflective of reduced motility) reduced TPH-2 expression. These changes in water content were associated for *L bulgaricus* with an increased paracellualr permeability that suggests that in addition to motor changes in paracellular permeability (fluid secretion) might contribute to increased motility (similarly to the action of linaclotide).

In conclusion, our study has identified single or groups of functionally relevant molecular targets that can be regulated specifically and differentially by bacterial strains. The ability of bacterial strains to induce transcriptomic regulation of key genes can serve as future therapeutical guidance. Therefore, development of such strategies can only go in parallel with the development of the knowledge of the physiopathologic process of diseases. However, it is in line with the development of personalized medicine and the treatment of symptoms more than diseases. Altogether our study also sets the basis for using combination of probitic strains to target “à la carte” multiple symptoms that are altered during GI or extra GI diseases.

Figures

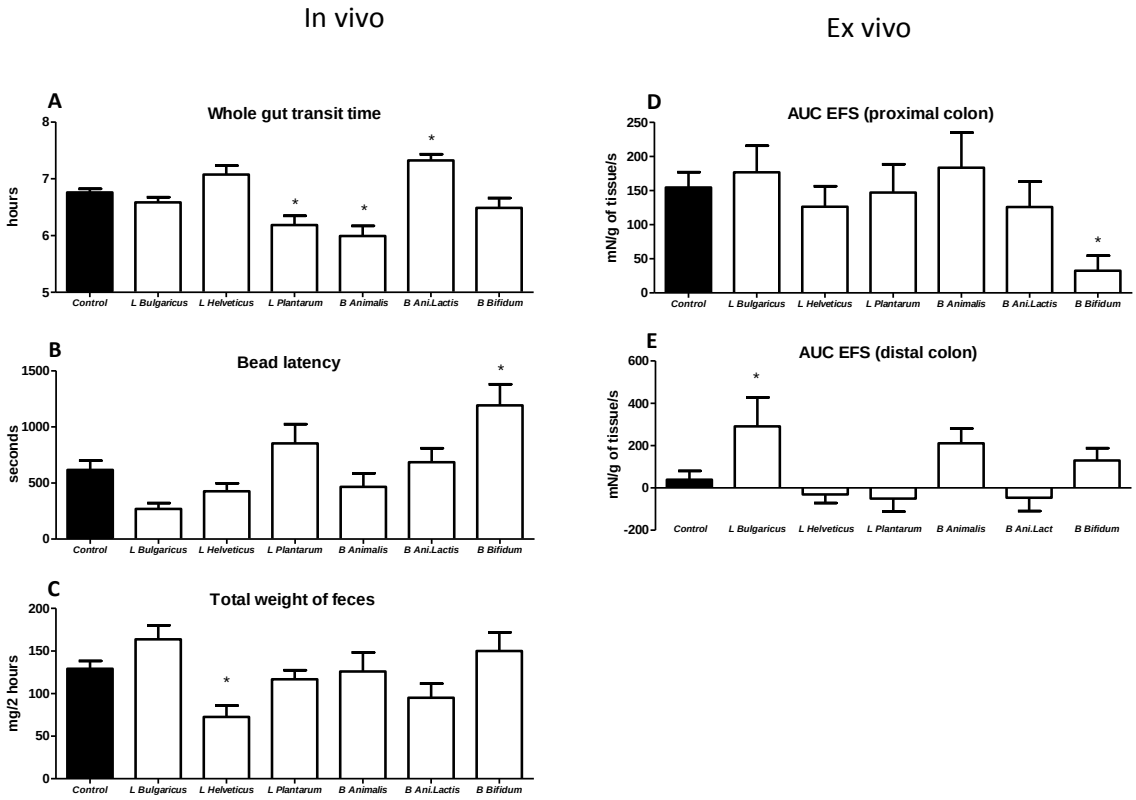

Figure 1: impact of bacterial strains on gastro-intestinal motility.

The gastro-intestinal motility was assessed both *in vivo* and *ex vivo*. After treatment, the whole gut transit time was increased with L *Plantarum*, B *Animalis* and decreased with B *Animalis lactis* (A). The distal colonic propulsive motility was also assessed *in vivo* measuring the bead latency time. Only B *Bifidum* increased significantly this time (B). The weight of faeces issued during 2 hours decreased after L *Helveticus* (C). The contractile response of colon strips after electrical field stimulation (EFS) of ENS was assessed *ex vivo* in organ bath. B *Bifidum* significantly decreased the area under the curve (AUC) of the contraction of the proximal colon (D) while L *Bulgaricus* increased AUC in the distal colon (E).

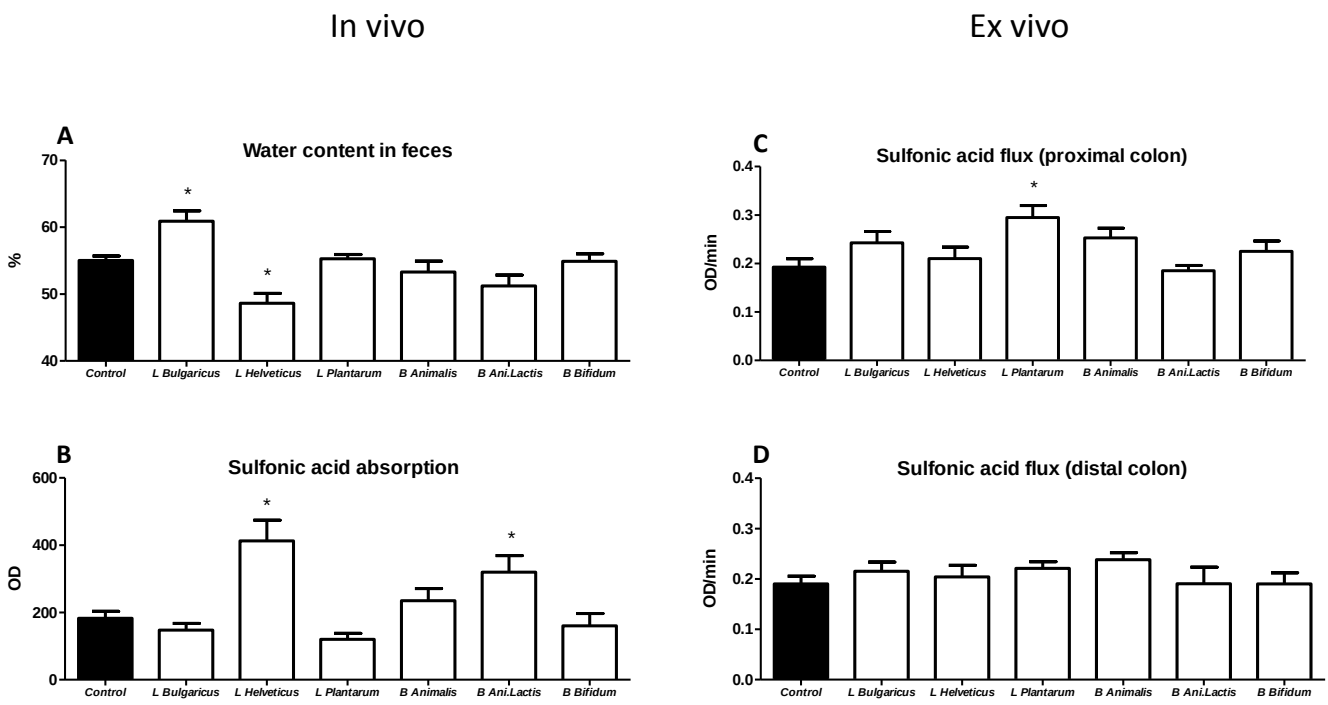

Figure 2: impact of bacterial strains on the gastro-intestinal permeability.

The water content of feces was determined by subtracting the weight of feces issued in 2 hours by the weight of feces dried. L *Bulgaricus* increased the percentage of water in feces while L *Helveticus* decreased (A). The paracellular permeability was assessed *in vivo* measuring the fluorescence of plasmas 1 hour after gavage of a sulfonic acid solution. L *Helveticus* and B *Animalis lactis* increased significantly the optical density (OD) of serum as a consequence of increased paracellular permeability (B). The paracellular permeability was also assessed *ex vivo* in Ussing chambers. It was increased with L *Plantarum* in the proximal colon (C) and unchanged in the distal colon (D).

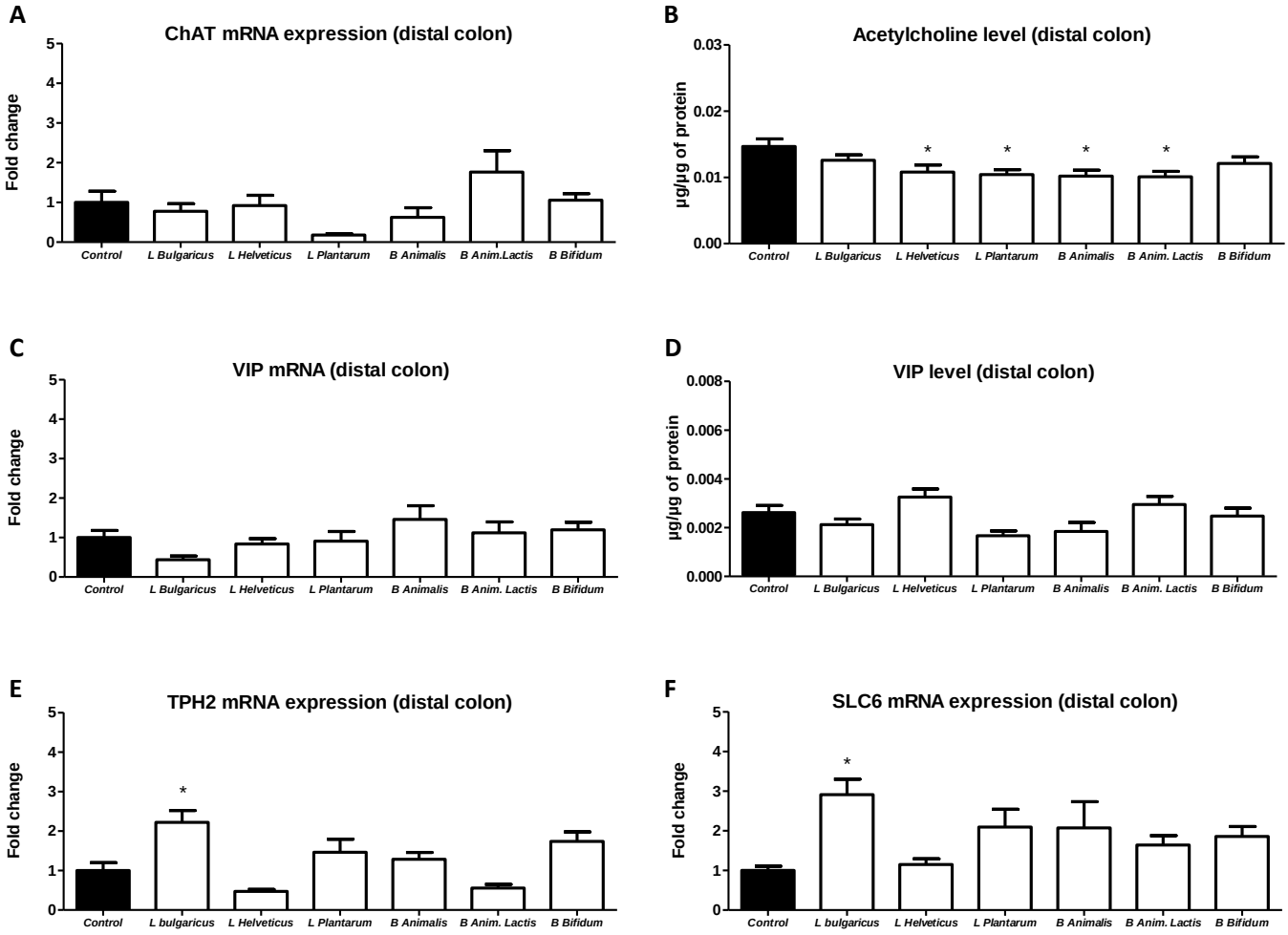

Figure 3: Impact of bacterial strains on neuronal markers in the distal colon

We did not observe any significant change of expression of ChAT mRNA or VIP mRNA in the distal colon (A, C). Acetylcholine amounts were decreased in the distal colon with L *Helveticus*, L *Plantarum*, B *Animalis*, B *Animalis lactis* (B) while VIP amounts were unchanged (D). TPH2 mRNA and SLC6 mRNA expressions were increased with L *Bulgaricus* (E, F).

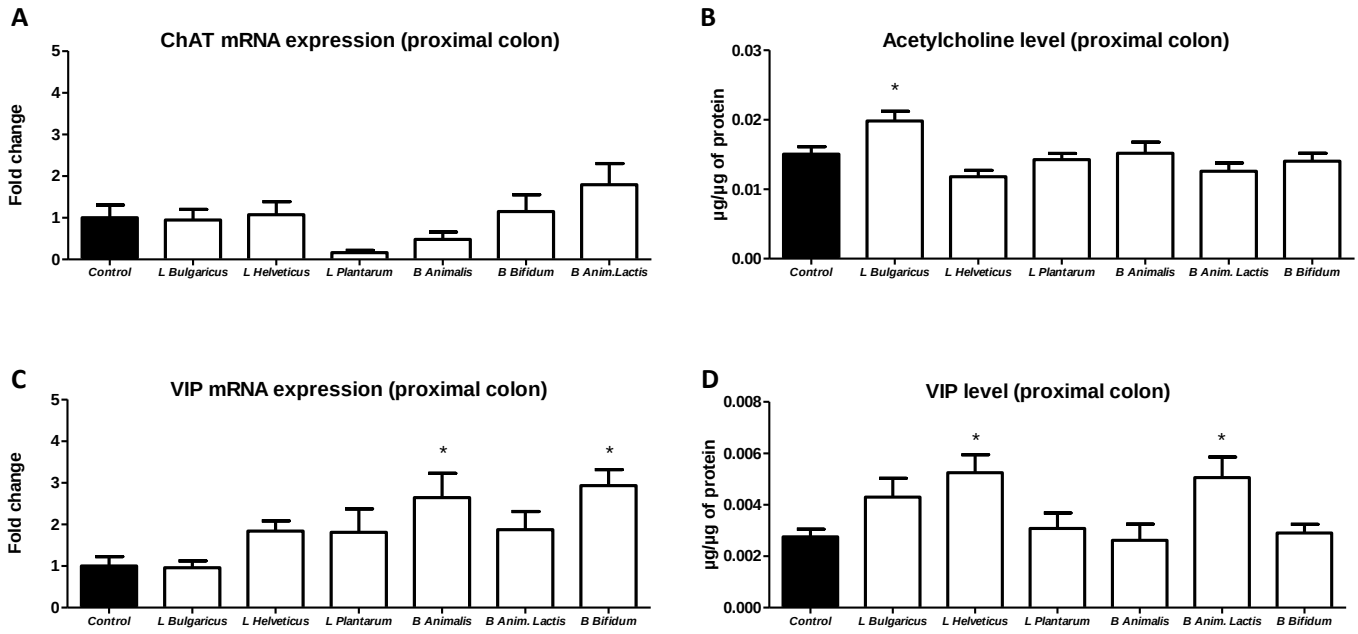

Figure 4: Impact of bacterial strains on neuronal markers in the proximal colon

We did not observe any significant change of expression of ChAT mRNA in the proximal colon (A). VIP mRNA expression was increased with B *Animalis* and B *Bifidum* in the proximal colon (C). Acetylcholine amounts were increased in the in the proximal colon with L *Bulgricus* (B). VIP amounts were also increased with *L Helveticus*, B *Animalis lactis* in the proximal colon (D).

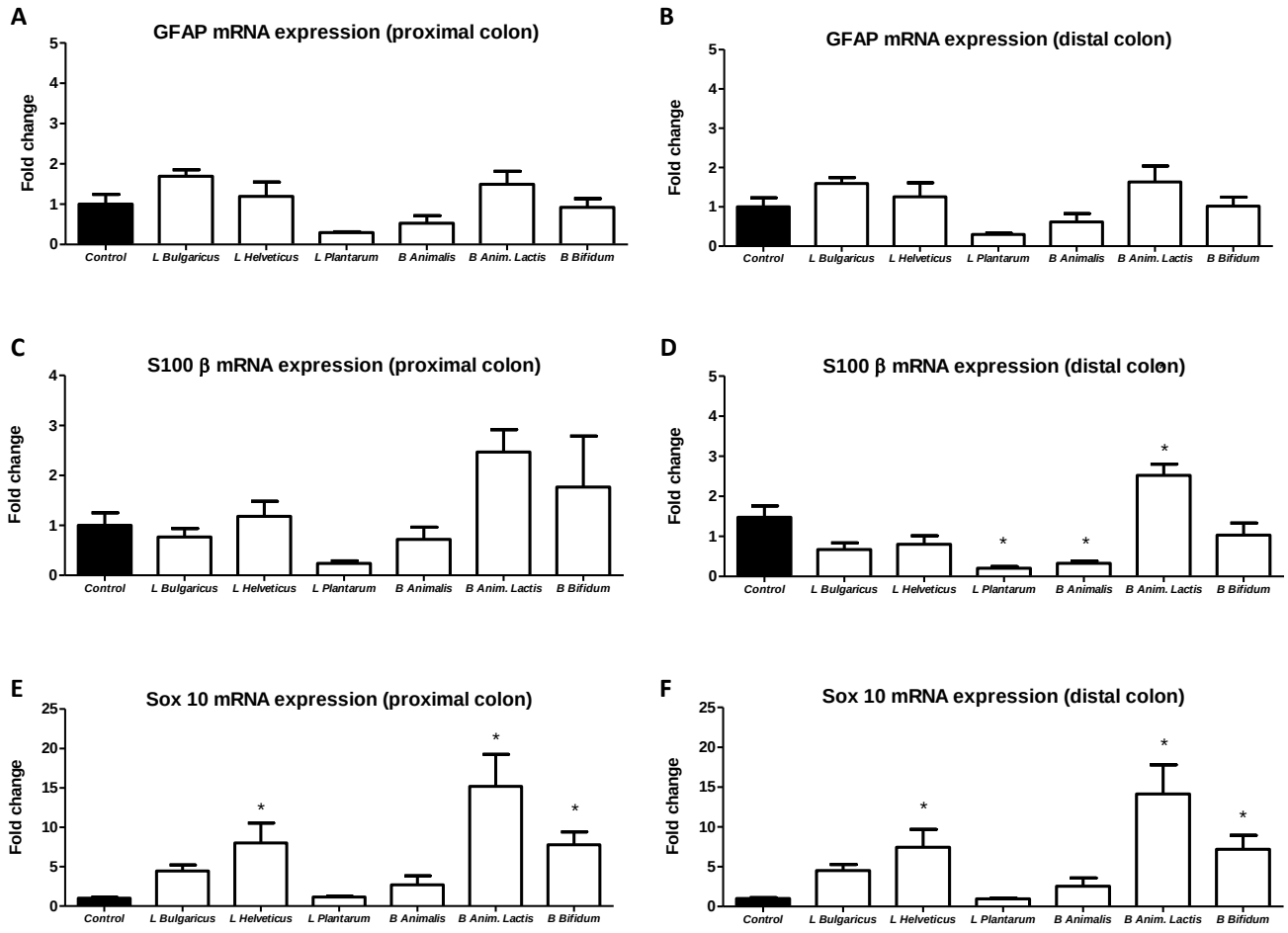

Figure 5: Impact of bacterial strains on enteric glial cells markers

GFAP mRNA expression was unchanged both in the proximal and distal colon (A,B). S100β m RNA expression was modified only in the distal colon (decreased by L *Plantarum* and B *Animalis* and increased by B *Animalis lactis*) (C,D). Sox 10 mRNA expression was increased both in the proximal and distal colon with L *Helveticus*, B *Animalis Lactis* and B *Bifidum* (E,F).

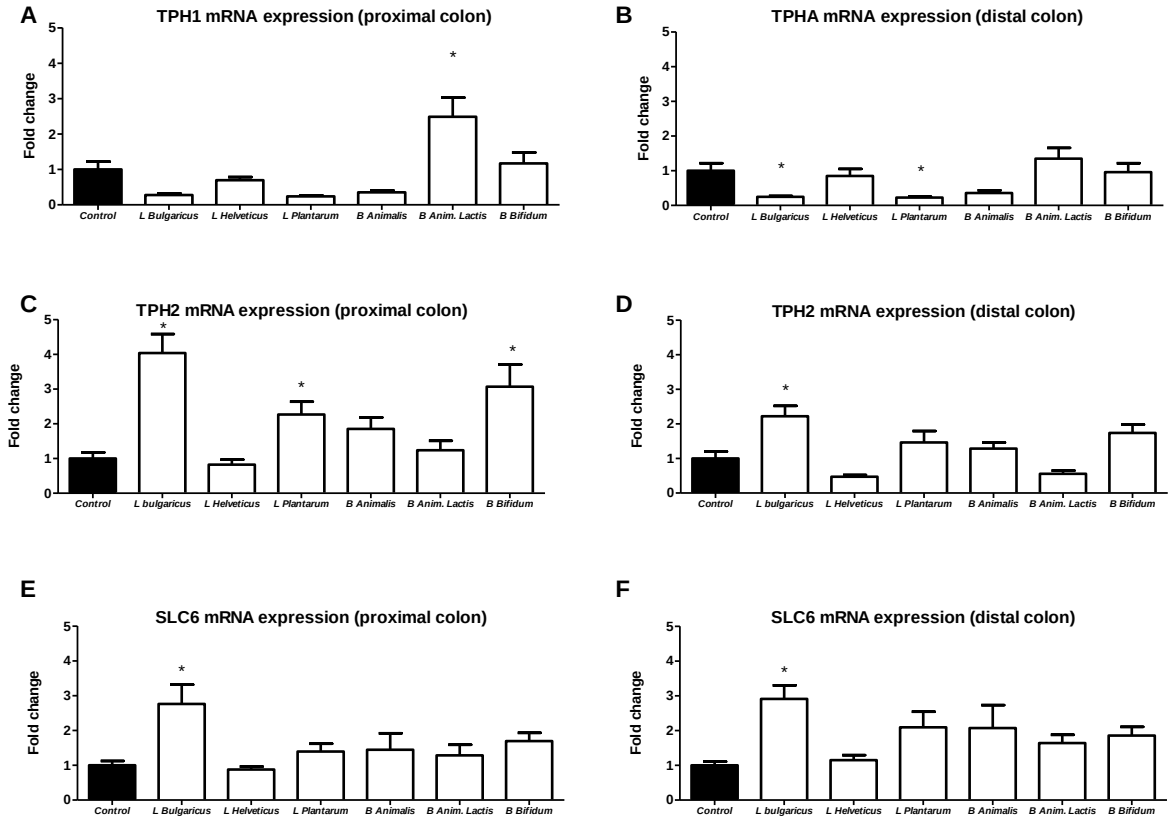

Figure 6: Impact of bacterial strains on the serotoninergic phenotype.

TPH1 mRNA expression was increased by B *animalis lactis* in the proximal colon (A) and decreased in the distal colon after L *plantarum* and L b*ulgaricus* (B). L *bulgaricus* increased TPH2 mRNA expression in both proximal and distal colon (C, D) and L *Plantarum* and B *bifidum* only in the proximal colon (D). Only L *bulgaricus* was able to increase mRNA expression of SLC6 in both proximal and distal colon (E, F).

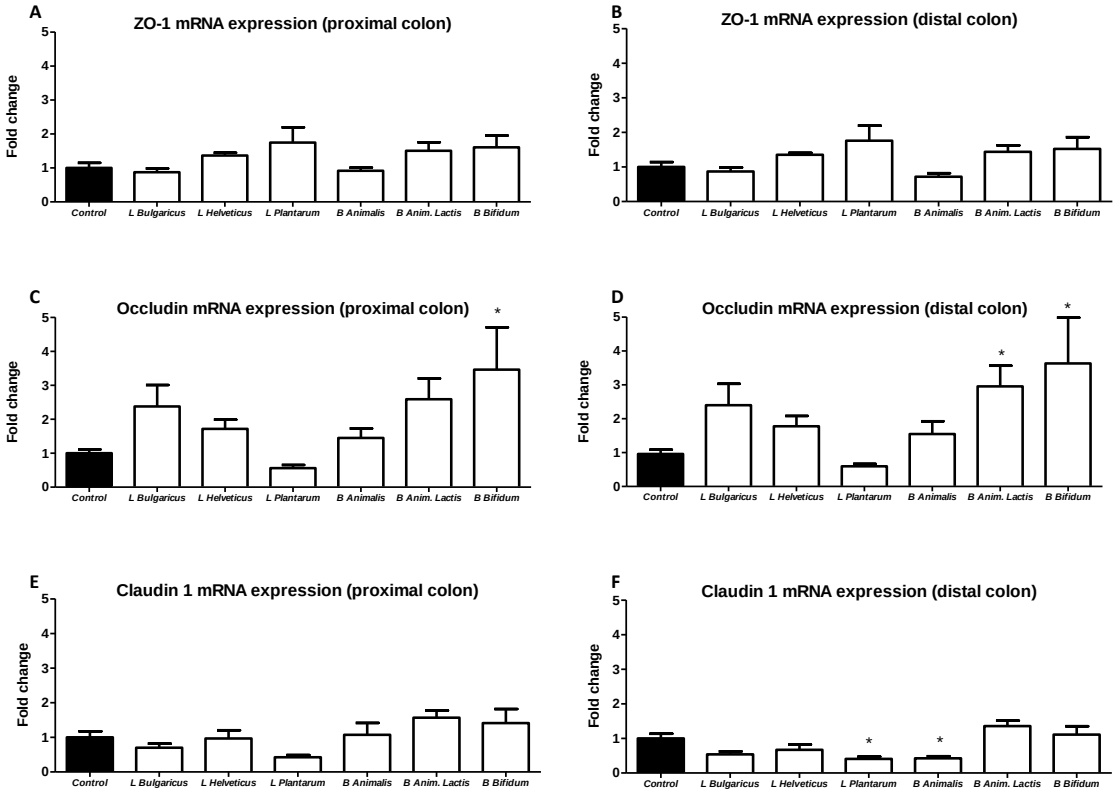

Figure 7: Impact of bacterial strains on junctional proteins.

ZO-1 mRNA expression was unchanged both in the proximal and distal colon (A, B). Occludin mRNA expression was increased in the proximal and distal colon with B *Bifidum* and with B *Animalis lactis* in the distal colon (C, D). Claudin 1 mRNA expression was only decreased in the distal colon by L *Plantarum* and B *Animalis* (E, F).

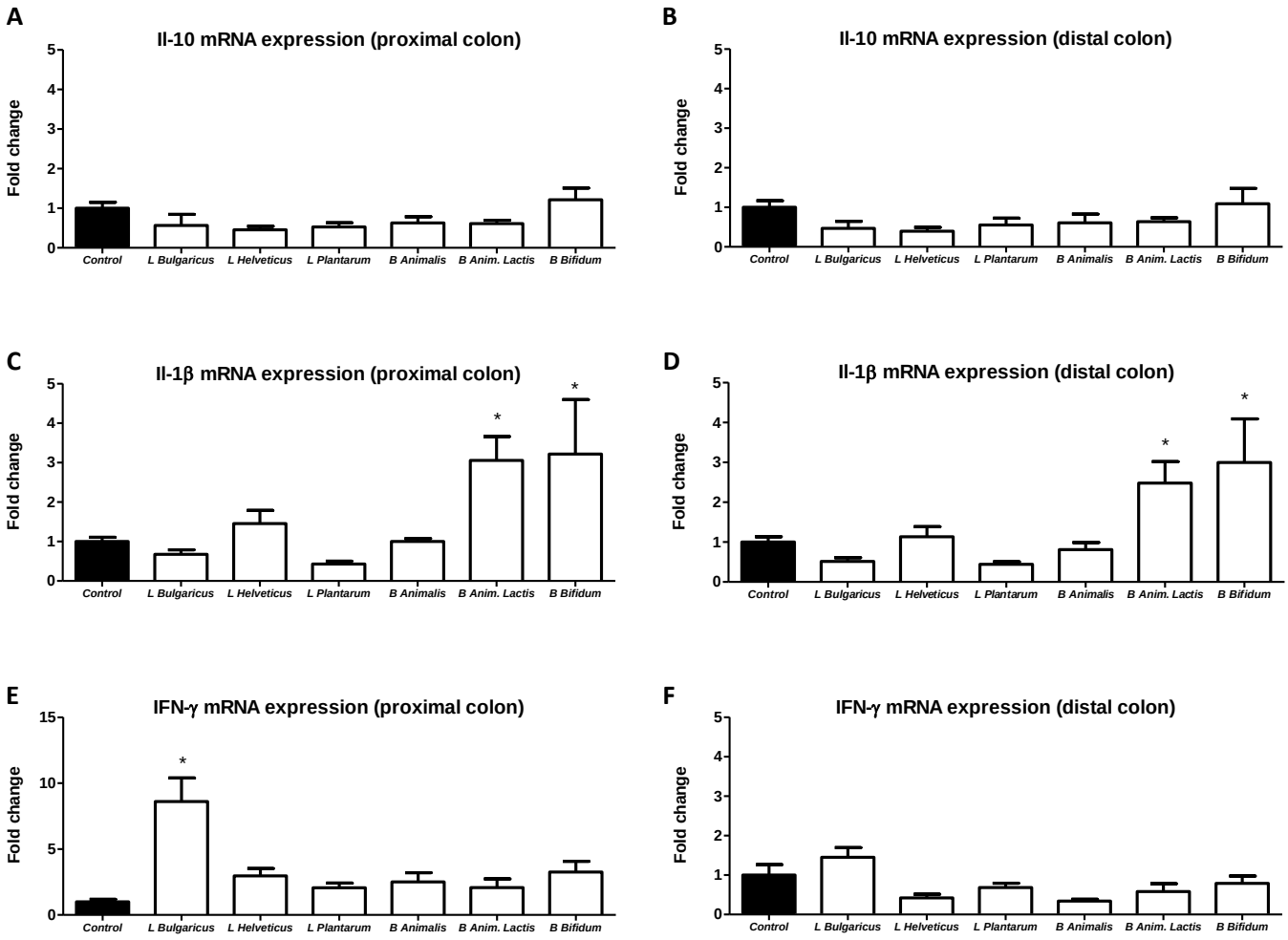

Figure 8: impact of strains on the colonic inflammation.

Il10 mRNA expression was unchanged both in the proximal and distal colon (A, B). Il-1β mRNA expression was increased by B *Animalis lactis* and B *Bifidum* in the proximal and distal colon (C, D). IFN-γ m RNA expression was increased only in the proximal colon with L *Bulgaricus* (E, F).

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Discussion générale

Ces travaux de thèse ont permis de 1) mieux caractériser les mécanismes physiologiques impliqués dans la maturation post-natale des fonctions digestives, en particulier le rôle du microbiote, 2) comprendre le rôle du microbiote dans la modulation du phénotype du SNE, et 3) proposer une approche thérapeutique innovante utilisant des souches bactériennes afin de moduler le phénotype neuro-glial et les fonctions digestives.

Pour ce faire, nous avons développé trois approches distinctes et complémentaires. Dans un premier temps, afin de mieux caractériser les mécanismes impliqués dans les troubles digestifs de la période postnatale et identifier le rôle du microbiote dans ces effets nous avons réalisé une étude observationnelle sur les facteurs de risques associés à la survenue des entérocolites ulcéro-nécrosantes et des perforations digestives isolées du prématuré. Dans un deuxième temps, nous avons voulu caractériser le rôle du microbiote sur la maturation du SNE et la mise en place des fonctions digestives. Dans cette optique, nous avons mis au point un modèle pré-clinique (raton nouveau-né) permettant d'évaluer l'impact du butyrate et de souches bactériennes d'intérêt dans ce contexte. Enfin, nous avons mis en place un protocole de recherche clinique prospectif visant à déterminer l'intérêt de lavements de butyrate sur la mise en place des fonctions digestives du nouveau-né prématuré.

Dans notre première approche, observationnelle, nous avons mis en évidence des facteurs associés à un risque accru de survenue d'entérocolites ulcéro-nécrosantes et de perforations intestinales isolées, pathologies digestives spécifiques des enfants prématurés. Un des résultats de cette étude est que l'usage d'une antibiothérapie empirique, en cas de risque d'infection materno-fœtale, semble être impliqué dans la survenue de ces pathologies. En effet, nous avons observé dans la cohorte nantaise de 3464 enfants prématurés que l'usage d'une antibioprophylaxie de moins de 3 jours était associé avec une baisse du risque de survenue d'entérocolite ulcéro-nécrosante. En revanche, un risque accru de perforation intestinale isolée en cas de traitement de 1 à 3 jours était observé. D'autres études ont montré un risque accru de survenue d'entérocolites ulcéro-nécrosantes en cas

d'infection maternelle comme la chorio-amniotite (Ragouilliaux *et al.* 2007; Been *et al.* 2013), de rupture prolongée des membranes (Sharma *et al.* 2010). Une autre étude a même souligné que le risque de survenue d'entérocolite ulcéro-nécrosante augmentait à chaque jour supplémentaire d'antibiothérapie néonatale (Cotten *et al.* 2009). De nombreux biais sont présents dans notre étude, en particulier son caractère rétrospectif mais concernent aussi les pratiques locales qui ont évoluées au cours du temps et qui diffèrent des autres centres. En effet, la proportion d'enfants prématurés exempts d'antibiothérapie empirique est variable en fonction des centres, de 2 à 16 % dans les études citées, à 37% dans notre étude. L'impact de l'antibiothérapie néonatale sur la composition du microbiote a été illustré par le suivi longitudinal de jumeaux nés prématurément où il a été montré une baisse initiale du nombre d'*E.coli* et/ou de *Klebsiella* dans les selles, réversible à l'arrêt des antibiotiques (Berrington *et al.* 2013). Plus précisément, il a été montré que l'antibiothérapie néonatale favorisait le développement d'*Escherichia* sp. et autres *Enterobacteriaceae* sp potentiellement impliquées dans la survenue d'entérocolites ulcéro-nécrosantes (Madan *et al.* 2012; Berrington *et al.* 2013). La composition du microbiote du méconium d'enfants atteints d'entérocolites présente également des particularités, notamment une augmentation du nombre de *Clostridium perfringens* et de *Bacteroides dorei*, alors que l'abondance de *Staphylococci* dans les selles post méconiales serait par contre un facteur protecteur (Heida *et al.* 2016). Une des voies de recherche dans la compréhension des mécanismes physiopathologiques impliqués dans la survenue d'entérocolite ulcéro-nécrosante serait alors de mieux identifier les phénomènes de dysbiose secondaires à l'usage précoce d'antibiotiques en période néonatale et permettre ainsi l'évaluation de l'effet des germes identifiés par notre modèle préclinique d'étude de la mise en place des fonctions digestives.

Dans un deuxième temps, nous avons voulu caractériser la capacité du microbiote et/ou de ses métabolites à moduler le phénotype du SNE et les fonctions digestives. Chez les animaux axéniques, l'absence de microbiote conduit à des modifications profondes des fonctions digestives notamment un ralentissement du temps de transit (Kashyap *et al.* 2013). Une des explications retrouvées est la présence d'anomalies du SNE (baisse du nombre de neurones, altération du phénotype neuro-chimique notamment la population nitrergique) (Collins *et al.* 2014). De manière intéressante, la restauration d'un microbiote a

permis de « normaliser » les fonctions digestives, en particulier la motricité tout comme la restauration d'un phénotype neuro-chimique du SNE (Kashyap *et al.* 2013; McVey Neufeld *et al.* 2013; Collins *et al.* 2014). L'utilisation d'animaux axéniques a également permis de tester l'effet de certaines souches bactériennes sur les fonctions digestives. En particulier, Husebye a montré qu'une colonisation digestive par *Lactobacillus acidophilus* A10 et *Bifidobacterium bifidum* B11 ou par *Clostridium tabificum* sp VP 04 induisait une baisse de la période de complexes moteurs migrants de l'intestin. Par contre, une colonisation par *E.Coli* X7 avait l'effet inverse, soit une augmentation de la période de complexes moteurs migrants de l'intestin (Husebye *et al.* 2001). Néanmoins, il est prudent d'émettre des réserves quant à la généralisation de tels résultats à la fois sur d'autres organes (jéjunum, côlon) et sur des animaux « conventionnels » (qui ont un tube digestif colonisé physiologiquement). En effet, Wu a montré sur des animaux conventionnels un effet de *Lactobacillus reuteri* (DSM 7938) différent sur les CMM du jéjunum et du côlon (fréquence abaissée ou augmentée respectivement) (Wu *et al.* 2013). Dans notre étude, nous avons mis en évidence la capacité de souches bactériennes à moduler différentiellement le phénotype neurochimique et glial du SNE du côlon ainsi que les fonctions digestives (motricité et perméabilité paracellulaire) sur un modèle animal « conventionnel ».

Les mécanismes impliqués dans les modifications observées restent à être explorés. Parmi les hypothèses mécanistiques, on retrouve l'effet d'éléments de la paroi bactérienne (lipopolysaccharides), qui pourraient être actifs même en cas de mort bactérienne. Un autre mode d'action pourrait être médié par la production de métabolites (acides gras à chaîne courte, lactacte). Enfin l'effet observé de certaines souches bactériennes pourrait être indirect, en favorisant la croissance de bactéries bénéfiques, ou au contraire en limitant la prolifération de bactéries pathogènes. Dans une étude *ex vivo*, Mao a montré que des fragments polysaccharidiques des membranes bactériennes pouvaient avoir un effet direct sur l'excitabilité des neurones (Mao *et al.* 2013). Les lipopolysaccharides bactériens (LPS) en agissant sur le TLR 4 augmentent la survie neuronale, notamment pour les neurones nitrergiques (Anitha *et al.* 2012). Enfin l'apport de bactéries lysées *(E. coli)* a permis de limiter les lésion de colites induites chez la souris traitée au dextran sodium sulfate (Konrad *et al.* 2003). Dans notre étude, nous n'avons pas vérifié la viabilité des bactéries testées dans les fèces des ratons mais nous avons gardé le matériel nécessaire pour une étude ultérieure.

Un autre mécanisme potentiel dans l'effet du microbiote sur le SNE et les fonctions digestives est le rôle des métabolites de la flore, en particulier les acides gras à chaîne courte. Parmi les acides gras à chaîne courte d'intérêt, le butyrate montre une concentration colique croissante dans les premières semaines de vie (Barrat *et al.* 2008). Cette concentration a même été augmentée par l'utilisation de prébiotiques (galactooligosaccharides), substrats des bactéries butyrogènes (Barrat *et al.* 2008). Les résultats présentés dans cette thèse sont en accord avec l'étude antérieure où il avait été montré chez le rat adulte que le butyrate induisait une augmentation de la proportion de neurones cholinergiques dans le plexus myentérique du côlon (Soret *et al.* 2010). De manière intéressante, nous avons également montré que les lavements de butyrate induisaient chez le raton une augmentation de la proportion de neurones myentériques nitrergiques. Cette réponse différentielle âge-dépendant pourrait être expliquée par une régulation différente des phénomènes épigénétiques en fonction de l'âge. En effet, il a été montré que l'action du butyrate sur le phénotype neuronal impliquait le transporteur MCT-2, spécifique, permettant la régulation de la transcription de l'ADN nucléaire par des mécanismes d'acétylation/desacétylation des histones (Soret *et al.* 2010). Une autre explication potentielle serait que le butyrate agisse sur la prolifération neuronale, comme montré dans le SNC (Kim, Leeds and Chuang 2009). Néanmoins cette hypothèse parait peu probable dans notre étude car le nombre de neurones par ganglion myentérique était similaire dans les différentes conditions expérimentales.

Outre son effet direct sur les neurones entériques, le butyrate agit également sur la muqueuse intestinale. Il représente une source d'énergie importante pour les cellules épithéliales intestinales, notamment au niveau du côlon où il est capté par les récepteurs GPR 41 et 43 (Karaki *et al.* 2008; Tazoe *et al.* 2009). De manière intéressante, les effets bénéfiques du butyrate sont dépendants de sa concentration. A concentrations physiologiques (dépendant de l'espèce, de l'organe et de l'âge), le butyrate a permis d'accélérer la cicatrisation d'anastomoses digestives chez le rat, de limiter les lésions de colites inflammatoires chez la souris, les pochites chez l'homme (Wischmeyer, Pemberton and Phillips 1993; Vernia *et al.* 2000; Bloemen *et al.* 2010; Mathew *et al.* 2010). A l'opposé, à de plus fortes concentrations (supérieurs à 200 mM), le butyrate a induit des lésions de colites chez le rat. Ces données sont consistantes avec les résultats observés sur les

fonctions digestives, où à doses physiologiques le butyrate a induit dans notre étude une accélération de la motricité propulsive du rectum du raton (et pas à dose supérieure), alors qu'à doses supérieures à 40 mM chez le rat adulte, a été observé une sensibilité accrue à la distension rectale (Lin *et al.* 2002; Bourdu *et al.* 2005; Nafday *et al.* 2005).

Cet effet « courbe en cloche » du butyrate pourrait avoir des conséquences physiologiques avec, à faibles concentrations un rôle protecteur, et à fortes concentrations un effet toxique. Ceci incite à la prudence quant à l'utilisation de butyrate dans le ciblage thérapeutique de pathologies digestives, en particulier l'entérocolite ulcéro-nécrosante où des concentrations coliques d'acides gras à chaînes courtes élevées ont été retrouvées. Ceci pourrait être expliqué par la présence de lésions muqueuses préalables, diminuant alors le transport de butyrate dans la cellule. Une autre explication serait la présence d'une dysbiose entraînant des phénomènes de fermentation et en conséquence la production excessive de métabolites délétères à l'homéostasie colique tels que l'acide lactique, le propionate, l'acétate.

Un résultat intéressant de notre étude est la capacité des bactéries à moduler le phénotype neuro-glial du SNE. Concernant la modulation du phénotype neuro-chimique du SNE nous avons montré pour la première fois *in vivo* la capacité de bactéries (*B bifidum* et *B animalis*) à réguler positivement le phénotype VIPergique des neurones entérique du côlon. De manière intéressante, *B animalis* avait montré dans le modèle *in vitro* de co-culture une réponse similaire. En revanche, l'effet de *L plantarum* montré *in vitro* n'a pas été retrouvé *in vivo*. Par ailleurs, les bactéries utilisées dans notre étude *in vivo* n'ont pas modulé l'expression de la ChAT attendue par le modèle *in vitro*. Une des explications potentielles serait que les modifications phénotypiques du SNE peuvent survenir sur l'ensemble du tube digestif alors que notre étude *in vivo* ne s'est focalisée que sur côlon. Une autre explication tiendrait du temps d'exposition insuffisamment court pour induire des modifications profondes du phénotype du SNE. Concernant la modulation de l'expression de marqueurs gliaux par les bactéries, nous avons montré une régulation positive de l'expression de S100β et de Sox 10 par *B animalis lactis* et *B bifidum* alors que *L plantarum* et *B animalis* ont entrainé une baisse d'expression de S100β au niveau du côlon distal. Le rôle du microbiote sur l'expression de marqueurs gliaux avait été étudié par Kabouridis qui avait montré qu'au décours de la colonisation de rats axéniques par une flore conventionnelle, l'expression de

marqueurs gliaux dans les villosités intestinales augmentait significativement (Kabouridis *et al.* 2015). Les conséquences d'une telle modulation restent à être explorées, notamment d'un point de vue fonctionnel.

Enfin, les travaux de cette thèse ont été valorisés par la mise en place d'une étude clinique interventionnelle dans le cadre d'un protocole hospitalier de recherche clinique (PHRC). Le protocole NEOTRANS vise à déterminer l'effet de lavements de butyrate de sodium (2.5 mM) sur la durée de nutrition parentérale, reflet du fonctionnement global des fonctions digestives. Il s'agit d'une étude monocentrique prospective en double aveugle. Le nombre d'inclusions est dicté par l'analyse triangulaire de groupes de 30 patients. A ce jour, 61 patients ont été inclus dans cette étude sans effet indésirable grave reporté. Un seul cas d'entérocolite ulcéro-nécrosante a été reporté, entrant dans le cadre de la prévalence attendue de cette maladie.

En conclusion, ce travail de thèse est une illustration de recherche translationnelle. Cette démarche pourrait être transposée dans le cadre d'autres pathologies caractérisées par des troubles de la motricité digestives comme la maladie de Hirschsprung, l'atrésie intestinale ou le laparoschisis. Dans ce contexte, l'étude des atteintes du SNE, en particulier les modifications du phénotype neuro-glial de ces pathologies, permettrait de proposer une démarche ciblée par l'utilisation du microbiote ou de ses métabolites.

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Annexes

Annexe 1: Postnatal development of myenteric neurochemical phenotype and impact on neuromuscular transmission in the rat colon (publication)

Annexe 2: Spontaneous intestinal perforation and necrotizing enterocolitis: a 16-year retrospective study from a single center (publication)

Annexe 3: Protocole de recherche clinique hospitalier NEOTRANS (PHRC NEOTRANS)

Annexe 1: publication

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

Auteurs : Philine de Vries, Rodolphe Soret, Etienne Suply, Yves Héloury, Michel Neunlist

Revue : American journal of physiology Gastrointestinal physiology

Année : 2010

Objectifs : étudier la maturation phénotypique des neurones myentériques du côlon chez le rat nouveau-né et les conséquences fonctionnelles.

Implication personnelle : J'ai participé à la réalisation des expériences *in vivo* et *ex vivo* ainsi qu'à l'analyse des données.

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

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de Vries P, Soret R, Suply E, Heloury Y, Neunlist M. Postnatal development of myenteric neurochemical phenotype and impact on neuromuscular transmission in the rat colon. *Am J Physiol Gastrointest Liver Physiol* 299: G539–G547, 2010. First published June 3, 2010; doi:10.1152/ajpgi.00092.2010.—Profound changes in intestinal motility occur during the postnatal period, but the involvement of the enteric nervous system (ENS), a key regulator of gastrointestinal (GI) motility, in these modifications remains largely unknown. We therefore investigated the postnatal development of the ENS phenotype and determined its functional repercussion on the neuromuscular transmission in the rat colon. Sprague-Dawley rats were euthanized at postnatal day (P) 1, P3, P5, P7, P14, P21, and P36. Whole mounts of colonic myenteric plexus were stained with antibodies against choline acetyltransferase (ChAT), neuronal nitric oxide synthase (nNOS), and HuC/D. Colonic contractile response induced by electrical field stimulation (EFS) was investigated in organ chambers in absence or presence of *N*-nitro-L-arginine methyl ester (L-NAME) and/or atropine. In vivo motility was assessed by measurement of the colonic bead latency time. Randomly occurring ex vivo contractions appeared starting at P5. Starting at P14, rhythmic phasic contractions occurred whose frequency and amplitude increased over time. In vivo, bead latency was significantly reduced between P14 and P21. Ex vivo, EFS-induced contractile responses increased significantly over time and were significantly reduced by atropine starting at P14 but were sensitive to L-NAME only after P21. The proportion of ChAT-immunoreactive (IR) neurons increased time dependently starting at P14. The proportion of nNOS-IR neurons increased as early as P5 compared with P1 but did not change afterward. Our data support a key role for cholinergic myenteric pathways in the development of postnatal motility and further identify them as putative therapeutic target for the treatment of GI motility disorders in the newborn.

postnatal development; enteric nervous system; myenteric plexus; rat; motility; neonates

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). 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 neuronally mediated contractile response in rat colon.

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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 $MgCl_2$, 1.2

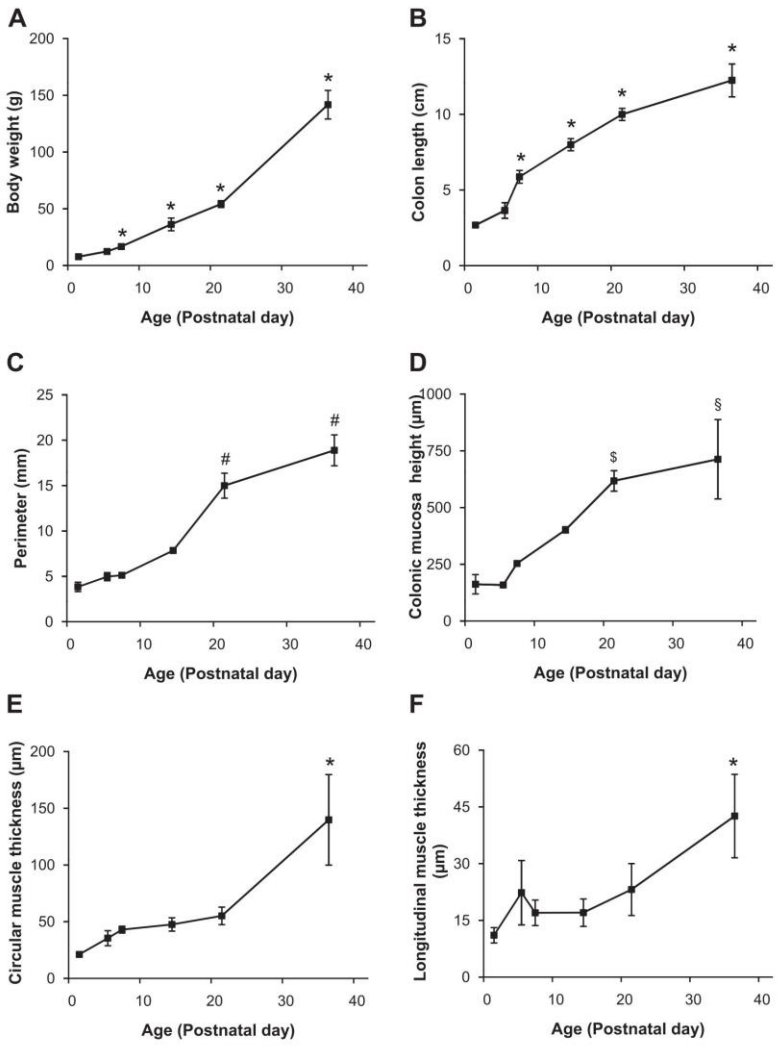

Fig. 1. Postnatal development of animal weight and colonic morphological parameters. Animal weight increased time dependently over the first 5 living weeks (*A*) (means ± SE; n = 10–20; 1-way ANOVA followed by Bonferroni's multiple-comparison test; $*P < 0.001$ compared with previous values). Colon length (*B*) and perimeter (*C*) increased over time [means ± SE; n = 4–6; 1-way ANOVA followed by Bonferroni's multiple-comparison test; $*P < 0.01$ compared with previous values; $\#P < 0.001$ compared with postnatal day (P) 14 and previous]. In parallel, mucosa height (*D*) and circular (*E*) and longitudinal (*F*) muscle thickness increased significantly starting at 3 wk of life (means ± SE; n = 4–6; 1-way ANOVA followed by Bonferroni's multiple-comparison test; $*P < 0.05$ compared with previous values; $\S P < 0.05$ compared with P7 value and previous values; $\$P < 0.05$ compared with P1 value).

NaH_2PO_4, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 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 nitrergic and cholinergic components of the EFS-induced contractile response, *N*-nitro-L-arginine methyl ester (L-NAME) 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% O_2-5% CO_2). 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 fluorimeter (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 1X for 3 h at room temperature. Tissues were then incubated sequentially with the primary antibodies for 3 h and the secondary antibodies for 1 h in the following order: goat anti-ChAT (1:200; Millipore) and anti-goat Cy3 (carboxymethylindocyanine) (1:500; Jackson ImmunoResearch); rabbit anti-nNOS (1:1,000; Alexis) and anti-rabbit Cy5 (7-amino-4-indodicarbocyanin) (1:500; Jackson ImmunoResearch) and mouse anti-HuC/HuD (1:200; Invitrogen) and anti-mouse FITC (fluorescein isothiocyanate) (1:500; Jackson ImmunoResearch). 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 of 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: $2\pi \cdot [(a^2+b^2)/2]^{1/2}$ 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. 1*A*) and the colon length from 2.7 ± 0.1 cm at P1 to 12.3 ± 1.1 cm at P36 (Fig. 1*B*). The perimeter of the proximal colon increased significantly over time starting the third postnatal week (Fig. 1*C*). Furthermore, circular and longitudinal muscles 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. 1*D*) 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

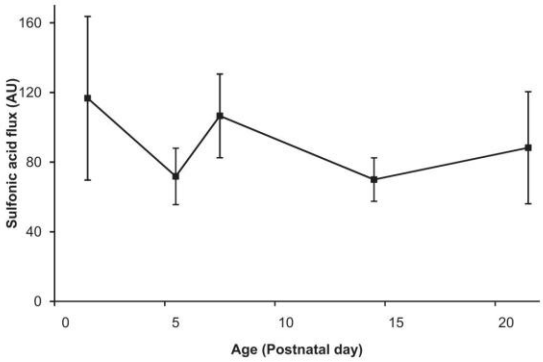

Fig. 2. Postnatal development of ex vivo paracellular permeability. The colonic permeability to sulfonic acid measured in Ussing chambers remained constant over the first 3 postnatal wk (means ± SE; $n = 4–6$; 1-way ANOVA followed by Bonferroni's multiple-comparison test). AU, arbitrary units.

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. 3*A*). 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. 3*B*) and amplitude (Fig. 3*C*) 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. 4*A*). Starting at P7, sparse individual ganglia could be detected (Fig. 4*B*). 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

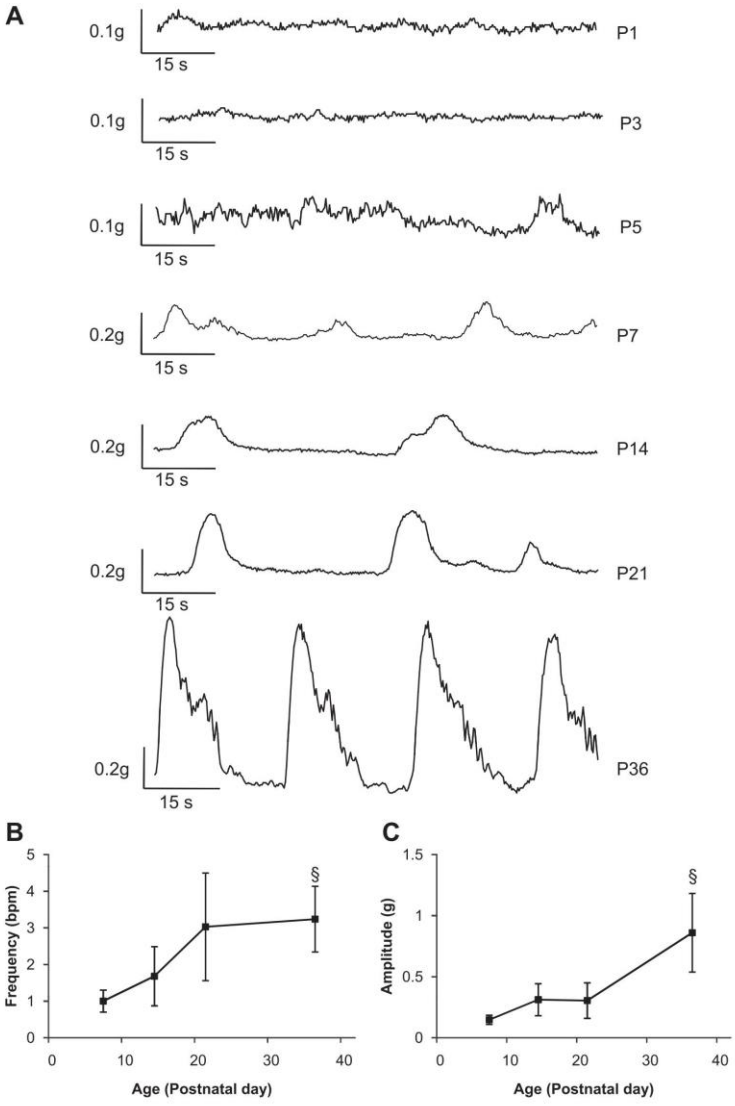

Fig. 3. Postnatal development of ex vivo spontaneous basal motility of colonic longitudinal muscle strips. At P1 (n = 8) and P3 (n = 4), no spontaneous contractile activity could be detected. At P5 (n = 12) few segments and at P7 (n = 8) 50% of the segments exhibited randomly occurring spontaneous contraction 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).

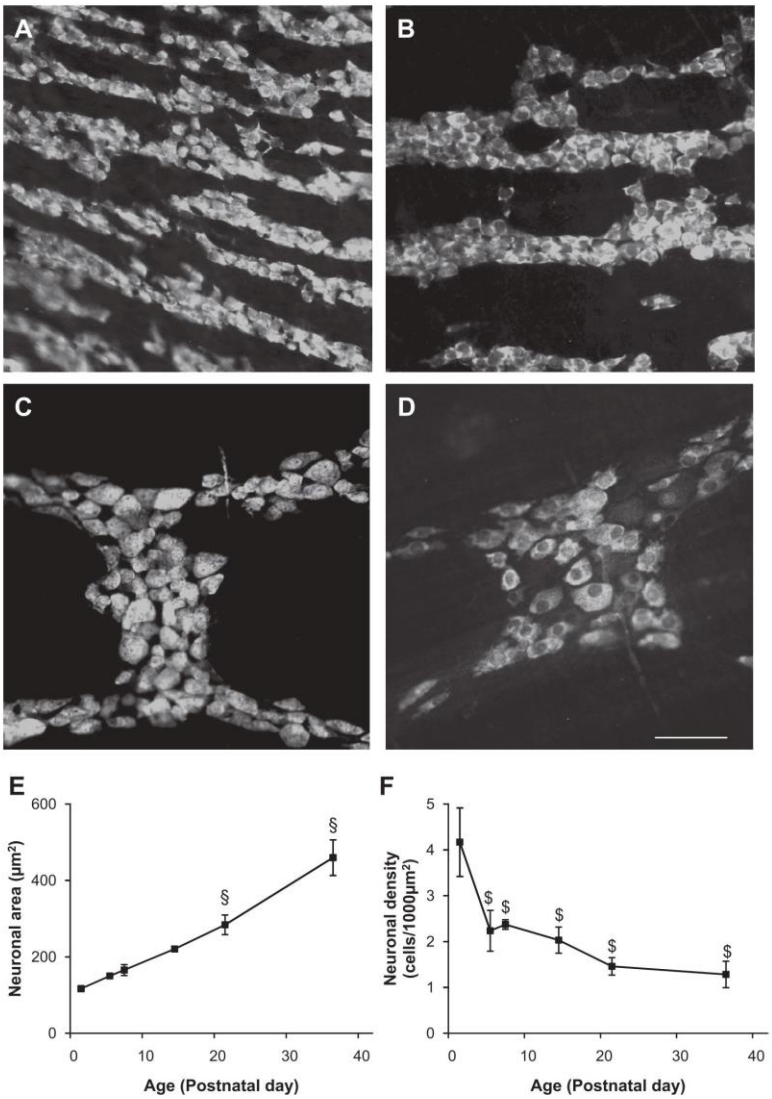

Fig. 4. Postnatal development of myenteric plexus organization and myenteric neurons morphology. Whole mount preparations of myenteric plexus of rat pup colonic specimens at P1 (*A*), P7 (*B*), P21 (*C*), and P36 (*D*) stained with a general neuronal marker Hu. At P1, neurons were organized in continuous rows parallel to the circular muscle. After P14, the enteric nervous system was organized as a network of individual ganglia connected by interganglionic fiber strands. Scale bar: 100 μm. Quantitative analysis showed a significant increase of neuronal surface area (*E*) and a decrease of cell density (*F*) over time (means ± SE; $n = 6$; 1-way ANOVA followed by Bonferroni's multiple-comparison test; §$P < 0.05$ compared with P7 and previous, $$P < 0.05$$ compared with P1) (scale bar = 100 μm).

time-dependent increase in neuronal cell surface area starting at P21 (Fig. 4*E*). In addition, neuronal cell density was significantly reduced as early as P7 compared with P1 (Fig. 4*F*).

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. 5*J*). 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. 5*K*). 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

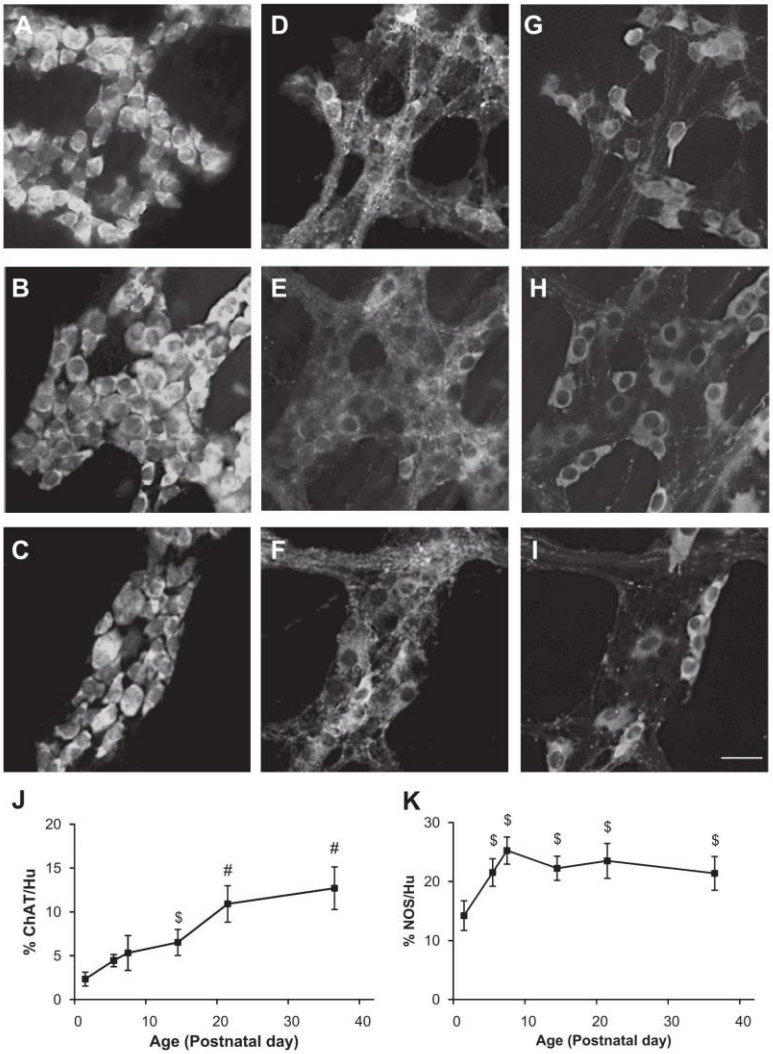

Fig. 5. Postnatal plasticity of the myenteric neurochemical phenotype. Triple immunohistochemical staining with antibodies against Hu (*A*, *B*, *C*), ChAT (*D*, *E*, *F*), and nNOS (*G*, *H*, *I*), on P7 (*A*, *D*, *G*), P14 (*B*, *E*, *H*), and P21 (*C*, *F*, *I*). Quantitative analysis showed a time-dependent increase in the proportion of ChAT-immunoreactive (IR) neurons (*J*) (normalized to Hu) during the postnatal period (means ± SE; $n = 6$; 1-way ANOVA followed by Bonferroni's multiple-comparison test; $\$P < 0.05$ compared with P1, $\#P < 0.05$ compared with P14 and previous). Quantitative analysis showed a time-dependent increase in the proportion of nNOS-IR neurons (*K*) only in the first postnatal week of life. (means ± SE; $n = 6$; 1-way ANOVA followed by Bonferroni's multiple-comparison test; $\$P < 0.05$ compared with P1) (scale bar = 50 μm).

presence of L-NAME and atropine (Fig. 6*A*). The EFS-induced AUC increased significantly over time starting at P14 compared with P1 but remained unchanged thereafter (2.3 ± 1.1 vs. 0.4 ± 0.2 g·s, respectively; $n = 6$ and 7, respectively) (Fig. 6*B*). In presence of L-NAME, EFS-induced AUC was significantly 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 neurons 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 excitatory pathways, of cholinergic (and probably also tachykinergic) 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,

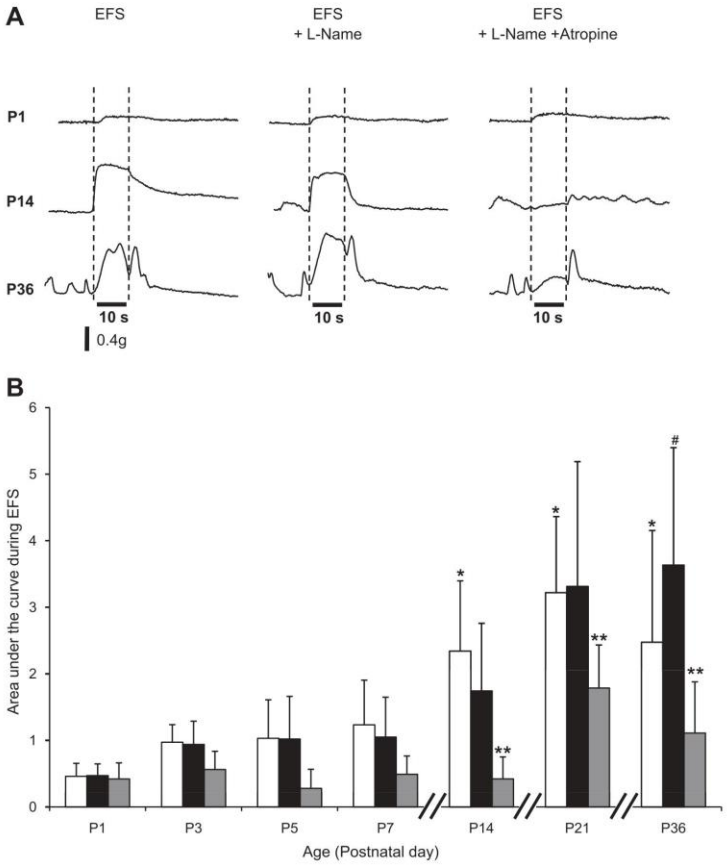

Fig. 6. Postnatal development of electrical field stimulation (EFS)-induced contractile response in the colon. Colonic longitudinal muscle strips were submitted to EFS and EFS-induced area under the curve (AUC) was analyzed in absence or in presence of *N*-nitro-L-arginine methyl ester (L-NAME) and atropine (*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).

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 occurs 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

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, Vannucchi 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 neuroplastic 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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GRANTS

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DISCLOSURES

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

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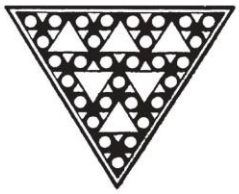

Annexe 2: publication

Titre : spontaneous intestinal perforation and necrotizing enterocolitis: a 16-year retrospective study from a single center

Auteurs : Etienne Suply, Marc-David Leclair, Michel Neunlist, Jean-Christophe Rozé, Cyril Flamant

Revue : European journal of pediatric surgery

Année: 2015

Objectifs : décrire la présentation clinique, la prise en charge et les conséquences de l'immaturité digestive des enfants prématurés qui se manifeste par l'entérocolite ulcéro-nécrosante et la perforation intestinale isolée.

Implication personnelle : le travail d'extraction et d'analyse de la base de données a été réalisé conjointement avec le Pr Rozé et le Dr Flamant. J'ai réalisé l'écriture de ce travail.

Original Article

Spontaneous Intestinal Perforation and Necrotizing Enterocolitis: A 16-Year Retrospective Study from a Single Center

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Eur J Pediatr Surg

Abstract

Background Necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (SIP) are serious abdominal conditions that affect preterm infants but are poorly understood. This single-center retrospective study was performed to identify the factors associated with NEC and SIP.

Methods This study involved 3,464 preterm infants recruited over 16 years. A total of 136 NEC and 24 SIP were analyzed and adjusted odds ratios (aOR) were determined by logistic regression.

Results Compared with the controls, NEC was associated with a Z-score for birth weight lower than −1 (aOR = 2.1 [1.1–3.9], $p = 0.02$) and lower than −2 (aOR = 4.4 [1.8–10.4], $p < 0.01$). NEC was associated with gestational ages of less than 31 weeks and with breech presentations (aOR = 1.5 [1.0–2.3], $p = 0.03$). In contrast, compared with the controls, SIP was associated with gestational ages of less than 26 weeks (aOR = 17.4 [3.1–96.2], $p < 0.001$) and multiple pregnancy (aOR = 2.9 [1.2–6.9], $p = 0.02$). Rates of mortality and cerebral lesions were higher in patients with NEC (25.0 and 13.2%, respectively) than with the controls (10.1 and 6.9%, respectively) and similar between patients with SIP (12.5 and 8.3%, respectively) and the controls.

Conclusion NEC and SIP differed in risk factors and prognosis. Early distinction of SIP from NEC could impact on surgical decision.

Keywords
- necrotizing enterocolitis
- spontaneous intestinal perforation
- risk factors
- very low-birth-weight infants

Introduction

Necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (SIP) are serious abdominal conditions that are associated with high morbidity in preterm infants. However, these diseases present significant clinical and pathophysiological differences.[1] NEC can be defined by the presence of an extensive inflammation and necrosis of the gastrointestinal track, in particular the colon, leading to sepsis and perforation. In contrast, SIP can be distinguished from NEC by a focal perforation due to a local necrosis of the gut wall. Indeed, according to Bell et al, NEC is a progressive disease,[2] which is usually diagnosed in patients with clinical deterioration, and sometimes in patients who develop radiological signs such as pneumatosis intestinalis. The pathophysiology of NEC is complex, and involves gut dysfunction and severe systemic degradation, in particular, through an exaggerated inflammatory response.[3] In contrast, SIP induces a sharp and clinical deterioration, and a sudden onset of pneumoperitoneum. Its pathophysiology remains poorly understood, and reports suggest the role of a weak muscular layer of the gut.[4]

Historically, surgical approaches for NEC and SIP have been similar, consisting of laparotomy or peritoneal drainage.

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However, since SIP was described as a different entity from NEC, reports had shown that the prognosis is also different.[5,6] The aim of this study was to describe the experience of a single center regarding the results and factors associated with NEC and SIP over a 16-year period.

Methods

All infants without congenital anomalies and born before 33 weeks of gestation in Nantes University Hospital (France) between January 1995 and December 2010 were included in the study.

All data were obtained from a prospective database, and all clinical observations of patients operated on for NEC or SIP were reviewed. NEC stage 2 or higher was defined based on Bell staging criteria for diagnosis.[2] As all infants presenting a pneumoperitoneum included in this study underwent a surgical procedure by laparotomy, patients with SIP were distinguished from patients with NEC based on the appearance of the bowel. In some cases, a needle puncture was performed as a first line of surgical approach. The needle puncture consisted of percutaneous puncture of the pneumoperitoneum with a 16- or 18-gauge needle, as described in a previous study,[7] and differed from classical peritoneal drainage.[8]

Empiric antibiotic therapy was initiated within the first hours of life and was classified into three categories: none, short ($\leq$ 2 days), or long ($>$ 3 days). Breech presentation included breech and transverse presentations. Prenatal steroid treatment was defined as at least one steroid injection. Persistent ductus arteriosus (PDA) was closed if clinically relevant, either medically (using indomethacin and ibuprofen before and after 2,000, respectively) or surgically, as a second-line therapy.

Univariate analyses were used to compare categorical variables (Chi-square test, two-tailed; significance level set at $p < 0.05$) or continuous variables (Student *t*-test, two-tailed; significance level set at $p < 0.05$). Two logistic regression models were used to determine the odds ratios (with 95% confidence intervals) for the association of NEC or SIP with risk factors computed as categorical variables (two-tailed tests; significance level set at $p < 0.05$). All analyses were performed with SPSS v.15.0 (Chicago, Illinois, United States).

Results

A total of 3,464 preterm infants were included in the study, including 136 infants with NEC (33 of whom had surgically treated NEC) and 24 infants with SIP. The overall mean gestational age (GA) was 29.5 $\pm$ 2.3 weeks, and the mean birth weight was 1.306 $\pm$ 429 g.

NEC incidence decreased over the time of the inclusion period (1995–1998: 9.0%, aOR = 1; 1999–2002: 4.0%, aOR = 0.5 [0.3–0.8], $p < 0.01$; 2003–2006: 2.2%, aOR = 0.3 [0.2–0.6], $p < 0.01$, 2007–2010: 1.9%, aOR = 0.2 [0.1–0.4], $p < 0.01$). Infants presenting with NEC had a mean GA of 28.2 $\pm$ 2.3 weeks and a mean birth weight of 1.022 $\pm$ 296 g. As indicated in ►Table 1, the univariate analyses revealed that infants with NEC were smaller than the control infants (lower GA and lower Z-scores for birth weight) and underwent significantly less prenatal corticosteroid treatment. The incidence of breech presentations was higher in infants with NEC than in the controls, but the rates of cesarean sections were similar between the two groups. PDA closure procedures (medical and surgical treatments), as well as umbilical catheterization, were significantly more common in infants with NEC than in the controls. Apgar score, the use of surfactant therapy, and catecholamine were similar between patients with NEC and the control group. Absent or long (but not short) empiric antibiotic treatment was more common in infants with NEC than in the controls. Multivariate analyses showed that NEC was associated with a shorter GA (<32 weeks), a Z-score for birth weight lower than -1 and -2, and breech presentation. NEC was inversely associated with empiric antibiotic treatment (►Table 2).

SIP incidence, in contrast, did not vary significantly over time (1995–1998: 0.6%, aOR = 1; 1999–2002: 0.3%, aOR = 1.1 [0.2–7.5], $p = 0.9$; 2003–2006: 0.4%, aOR = 2.4 [0.4–14.4], $p = 0.3$; 2007–2010: 1.0%, aOR = 5.4 [0.6–45.6], $p = 0.1$). Infants presenting with SIP had a mean GA of 27.3 $\pm$ 2.7 weeks and a mean birth weight of 904 $\pm$ 241 g. The univariate analyses (►Table 1) showed that infants with SIP had a lower GA than the control infants. No association was found with birth weight. As compared with the controls, infants with SIP underwent significantly more medical PDA closure procedures, more catecholamine treatment, venous umbilical catheterization, and prolonged empiric antibiotic treatment. The logistic regressions performed showed that SIP was associated with a GA of less than 26 weeks, multiple pregnancies, and prolonged empiric antibiotic treatment (►Table 2).

Medical reports were obtained in 28 cases of surgically treated NEC and all cases of SIP. The initial surgical procedure was performed later in patients with NEC than in patients with SIP (21.6 $\pm$ 13.9 days vs. 7.6 $\pm$ 5.8 days) and consisted of laparotomy for most of the NEC cases (96.4%, $n = 27$) and half of the SIP cases (50.0%, $n = 12$). The primary alternative procedure was needle puncture, which was performed for half the number of infants in the SIP group (50.0%, $n = 12$) and only one patient with NEC (3.6%, $n = 1$). Needle puncture was only repeated in patients with SIP (2 times for 6 patients and 3 times for 1 patient). In all cases, the needle puncture was unsuccessful, leading to recurrent pneumoperitoneum and/or clinical deterioration secondary to sepsis. Thus, laparotomy was performed 5 days after needle puncture in patients with NEC and 10.4 $\pm$ 12.0 days after needle puncture in patients with SIP. Intestinal perforations were observed in 67.9% ($n = 19$) of infants with NEC, and multiple intestinal perforations were observed in 10.7% ($n = 3$) of these patients. In SIP infants, multiple perforations were observed in 12.5% ($n = 3$), always a few centimeters from each other. Perforations were observed in jejunum ($n = 2$), ileum ($n = 20$), ascending colon ($n = 1$), and descending colon ($n = 1$).

The surgical approach for NEC and SIP (►Table 3) consisted mainly of intestinal resection with stoma for NEC. For SIP, perforation was exteriorized as a stoma in main cases. The

Table 1 Characteristics of infants with necrotizing enterocolitis (NEC) or spontaneous intestinal perforation (SIP) compared with controls

	Controls		NEC			SIP		
	n = 3,306		*n* = 136			*n* = 24		
	n	(%)	*n*	(%)	*p*	*n*	(%)	*p*
Infant characteristics								
Sex (male)	1,831	(55.4)	73	(53.7)	0.69	10	(41.7)	0.67
Gestational age (wk)								
24–25	196	(5.9)	16	(11.8)	<0.001	9	(37.5)	<0.001
26–27	465	(14.1)	46	(33.8)		6	(25.0)	
28–29	737	(22.3)	30	(22.1)		3	(12.5)	
30–31	1,108	(33.5)	31	(22.8)		4	(16.7)	
> 32	800	(24.2)	13	(9.6)		2	(8.3)	
Z-score for birthweight								
< -2	87	(2.6)	10	(7.4)	0.001	2	(8.3)	0.39
−2; −1.01	578	(17.5)	33	(24.3)		3	(12.5)	
−1; −0.51	565	(17.1)	25	(18.4)		5	(20.8)	
-0.5; +0.5	1,531	(46.3)	47	(34.6)		9	(37.5)	
> +0.5	545	(16.5)	21	(15.4)		5	(20.8)	
Pregnancy and birth characteristics								
Multiple pregnancy	985	(29.8)	30	(22.1)	0.06	11	(45.8)	<0.001
Diabetes	135	(4.1)	3	(2.2)	0.27	1	(4.2)	–
Hypertension	560	(16.9)	21	(15.4)	0.65	2	(8.3)	0.26
Prenatal corticosteroid treatment	2,042	(61.8)	71	(52.2)	0.02	16	(66.7)	0.62
Outborn status	552	(16.7)	26	(19.1)	0.46	6	(25.0)	0.28
Cesarean section	2,002	(60.6)	79	(58.1)	0.56	15	(62.5)	0.85
Compound presentation	712	(21.5)	40	(29.4)	0.03	4	(8.3)	0.56
Neonatal characteristics								
Apgar < 4 (1 min)	558	(16.9)	30	(22.1)	0.12	5	(20.8)	0.61
Surfactant therapy	1,415	(57.2)	68	(50.0)	0.1	13	(54.2)	0.26
PDA treatment								
Medical	377	(11.4)	24	(17.6)	0.03	8	(33.3)	<0.001
Surgical	77	(2.3)	8	(5.9)	0.009	1	(4.2)	–
Medical and surgical	416	(12.6)	28	(20.6)	0.006	8	(33.3)	0.002
Catecholamine treatment	129	(3.9)	7	(5.1)	0.46	9	(47.5)	0.001
Umbilical artery catheterization	80	(2.4)	13	(9.6)	<0.001	1	(4.2)	–
Umbilical vein catheterization	1,837	(55.6)	108	(79.4)	<0.001	18	(75.0)	0.06
No therapy	164	(5.0)	10	(7.3)	0.21	5	(20.8)	<0.001
Empiric antibiotic treatment								
None	732	(22.1)	50	(36.8)		4	(16.7)	
≤ 2 d	2,067	(62.5)	60	(44.1)	<0.001	8	(33.3)	<0.001
> 3 d	507	(15.3)	26	(19.1)		12	(50.0)	

intestinal resection rate was higher and involved a longer intestinal segment in patients with NEC than in patients with SIP. Similar rates of intestinal stoma were observed in both groups.

The rate of postoperative complication was similar between patients with NEC and SIP (17.9 vs. 13.6%). For patients with NEC, these complications included two wound abscesses, a wound dehiscence, a stoma prolapse, and two cases of

Table 2 Risk factors for necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (SIP) determined by comparison with control infants ($n = 3,306$)

	NEC ($n = 136$)			SIP ($n = 24$)		
	aOR	95% CI	*p*	aOR	95% CI	*p*
Infant characteristics						
Z-score for birthweight						
< -2	4.38	(1.85–10.38)	<0.001	5.36	(0.82–35.13)	0.08
$-2; -1.01$	2.12	(1.14–3.95)	0.02	1 0.00	(0.21–4.78)	1
$-1; -0.51$	1.56	(0.83–2.91)	0.17	0.75	(0.16–3.42)	0.71
$-0.5; +0.5$	0.98	(0.57–1.67)	0.94	0.74	(0.24–2.31)	0.6
$> +0.5$	1			1		
Gestational age (wk)						
24–25	5.49	(2.5–12.05)	<0.001	17.41	(3.15–96.18)	<0.001
26–27	6.91	(3.64–13.1)	<0.001	3.72	(0.69–20.23)	0.13
28–29	2.95	(1.51–5.77)	<0.001	1.37	(0.22–8.53)	0.74
30–31	2.09	(1.08–4.06)	0.03	1.51	(0.27–8.45)	0.64
> 32	1			1		
Sex (male)	0.98	(0.68–1.39)	0.89	1.13	(0.47–2.73)	0.79
Pregnancy and birth characteristics						
Multiple pregnancy	0.71	(0.46–1.08)	0.11	2.86	(1.18–6.88)	0.02
Hypertension	0.64	(0.38–1.11)	0.11	0.46	(0.09–2.34)	0.35
Compound presentation	1.55	(1.04–2.31)	0.03	0.3	(0.07–1.33)	0.11
Cesarean section	0.74	(0.49–1.13)	0.16	2.23	(0.81–6.18)	0.12
Neonatal characteristics						
Empirical antibiotic treatment						
None	1			1		
≤ 2 d	0.43	(0.29–0.65)	<0.001	1.04	(0.26–4.1)	0.96
> 3 d	0.56	(0.33–0.94)	0.03	4.13	(1.04–16.52)	0.05

secondary intestinal necrosis. In patients with SIP, postoperative complications were observed only after needle puncture and consisted of wound dehiscence ($n = 1$), stomal stenosis ($n = 1$), and a digestive fistula ($n = 1$).

The mortality rate was 10.1% in the control group ($n = 333$), 25.0% in patients with NEC ($n = 34$; $p < 0.05$ vs. controls), 33.3% in surgical NEC cases ($n = 11$; $p < 0.05$ vs. controls), and 12.5% in patients with SIP ($n = 3$; $p = 0.9$ vs. controls). The rate of cerebral lesions (intraventricular hemorrhagia grade 3 or higher and periventricular leucomalacia) was 6.9% ($n = 228$) in the control group, 13.2% in patients with NEC ($n = 18$; $p < 0.05$ vs. controls), 12.1% in surgical NEC cases ($n = 4$; $p < 0.05$ vs. controls), and 8.3% in patients with SIP ($n = 2$). The time before stoma closure in surviving infants was similar after NEC (80.2 ± 34.4 days) or SIP (73.9 ± 28.4 days). Hospital stay of survival was 44.4 ± 31.0 days in controls, 77.5 ± 40.7 101 days in NEC ($p < 0.01$ vs. control), 101.8 ± 55.4 days in surgical NEC ($p < 0.01$ vs. control), and 84.1 ± 51.6 days in SIP ($p < 0.05$ vs. control).

Discussion

The present study revealed that the incidence of NEC strongly decreased over the 16-year period, while the incidence of SIP remained stable. We hypothesize that progress made in neonatal care during this period (improvement of pulmonary disease management, feeding practices, and hospital hygiene) may have had an impact on the incidence of NEC, but not SIP. Regarding feeding practices, minimal enteral feeding and greater use of human milk for very preterm infants (systematic administration in our unit until 32 weeks of gestational age and/or weight > 1500 g) could explain this diminution of incidence of NEC. In return, the stable incidence of SIP could be due to an inherent vulnerability of preterm infants.

Surgical intervention for SIP most often occurred earlier in life than surgery for NEC, which suggests that the mechanisms involved are different. Intestinal perforation observed in SIP could be the consequence of a weak gut and occurs before the enteral feeding volume has been established.[9]

Table 3 Surgical characteristics of infants presenting necrotizing enterocolitis (NEC) or spontaneous intestinal perforation (SIP)

	NEC ($n = 28$)		SIP ($n = 24$)	
Postnatal age at surgery, days ± SD	21.6	± 13.9	7.6	± 5.8
Bowel perforation, % (*n*)	67.9	(19)	100.0	(24)
Primary surgery				
Puncture, % (*n*)	3.6	(1)	50.0	(12)
Laparotomy % (*n*)	96.4	(27)	50.0	(12)
Successful puncture, % (*n*)	0.0	(0)	0.0	(0)
Time between puncture and surgery, days (± SD)	5.0		10.4	± 12.0
Surgical procedure				
Stoma with the perforation, % (*n*)	28.6	(8)	66.7	(16)
Suture of the perforation, % (*n*)	7.1	(2)	16.7	(4)
Resection and stoma, % (*n*)	57.1	(16)	12.5	(3)
Resection and anastomosis, % (n)	3.6	(1)	4.2	(1)
Proximal enterostomy, % (*n*)	7.1	(2)	4.2	(1)
Resection, % (*n*)	60.7	(17)	16.7	(4)
Length of resection, cm (± SD)	9.9	± 10.8	7.0	± 5.2

Interestingly, histological studies of SIP report segmental defects of the circular muscular layer of the gut adjacent to the perforation as a consequence of gut immaturity.[4] In contrast, surgical procedures for NEC occurred later in life, usually after a few days of development, which suggest that multiple factors such as robust inflammatory processes are involved in the genesis of NEC.[3]

The incidence of NEC was inversely correlated with GA and the Z-score for birth weight. In contrast, SIP mostly affected infants born before 27 weeks of gestation, and was not associated with birth weight. Thus, the risk of developing SIP strongly decreases after 28 weeks of gestation, while the risk of developing NEC decreases progressively with term, although the risk remains for older infants. Finally, SIP, but not NEC, was associated with multiple pregnancies, underlying the potential effect of placental dysfunction and growth disturbances in SIP pathogenesis.[10]

Interestingly, no association between SIP or NEC and medical treatment for PDA was noted in the present study. In contrast, previous studies reported that early treatment with indomethacin (either at <12 hours of life[11] or before day-3 of life[5]), could be associated with a higher incidence of SIP and a lower incidence of NEC. This discrepancy could be due to delayed administration of treatment (after day-2) in our institution and the lower intestinal morbidity induced by the use of ibuprofen instead of indomethacin.[12]

Higher rates of NEC and SIP have been described for cases of chorioamniotitis[13,14] or prolonged rupture of membranes (>24 hours),[11] leading to prolonged antibiotic treatment, which is also associated with NEC.[15] In our study, NEC was associated with an absence of antibiotic treatment, while initial empiric antibiotic treatments of less than 3 days seemed to be a protective factor in this retrospective study. This result contrasts with what has been reported in other studies[15,16]; that is, there was an association between the incidence of NEC and previous antibiotic treatment. However, in these studies, the proportion of infants without empirical antibiotic therapy within the first three days of life was between 2 and 16%, while in our study the proportion was 37%, indicating that there is a high discrepancy between centers. This result should be confirmed in further prospective studies.

The needle puncture procedure, which is based on the high capacity of the newborn gut to heal, was not effective in our study, in contrast to a previous report.[7] Consequently, we do not recommend this procedure. This could be because we punctured mainly the pneumoperitoneum and rarely observed liquid outpouring, which makes the procedure more similar to peritoneal drainage, which is widely described. Peritoneal drainage was not used in this monocentric retrospective study, but may be of interest, particularly for rescue procedures. This study also reported a large proportion of stoma for treating SIP. Creating digestive stoma appears to be a quick and safe procedure for these frail infants. However, this result should be linked to a survival rate and a cerebral prognosis similar to those observed in the control group, even though the SIP infants were smaller. Primary anastomosis may be preferable, but the results need to be confirmed in future investigations. Concerning NEC, we performed intestinal resection in 60% of cases with an average length of 10 cm, sparing the gut as much as possible. We speculate that larger but still reasonable resections could result in a better neurological prognosis, minimizing the inflammatory process and the systemic response.

Our study had several limitations. First, the management of infants has changed during the study period. If the long period (16 years) of recruitment allows to appreciate the

evolution of incidence of NEC and SIP, infants management is very different and must be taken into consideration. Moreover, although this management follows international guidelines, a few medical procedures are not consensual, as for patent ductus arteriosus or empiric antibiotic treatment, and must be considered as a limitation of our study. These treatments are particularly used in the population of very low-birth-weight infants that could represent a possible source of confounding and bias.

In conclusion, we have shown in this study that the incidence of NEC decreased over the recruitment period, while the incidence of SIP remained stable. The risk of developing NEC increased as GA and the birth weight Z-score decreased, whereas the risk of developing SIP was only increased for extremely preterm infants (GA $<$ 26 weeks). Peritoneal puncture failed in all cases of SIP, prompting our institution to give up this procedure. The effect of initial empiric antibiotic therapy of less than 3 days on the incidence of NEC should be more closely studied.

Conflict of Interest

None.

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Annexe 3 : Protocole de recherche NEOTRANS

Titre : Effects of Recto-colic Enemas of Butyrate on the Digestive Disorders of Very Low Birth Weight Preterms <1250 Grams. Clinical Trial Prospective, Monocentric, Randomized in Double-blinded.

Auteurs : Etienne Suply, Marc-David Leclair, Michel Neunlist, Arnaud Legrand, Jean-Christophe Rozé, Cyril Flamant

Année : en cours

Objectifs : déterminer les effets de lavement de butyrate sur la tolérance alimentaire et la mise en place de la motricité digestive chez les nouveau-nés prématuré de poids de naissance inférieur à 1250 grammes.

Implication personnelle : la conception de l'étude, l'écriture du protocole, les démarches administratives ont été réalisées conjointement avec le CIC-04 de pédiatrie, Jean-Christophe Rozé, Cyril Flamant, Arnaud Legrand.

Extrait de la base Clinical trial

Title:

Effects of Recto-colic Enemas of Butyrate on the Digestive Disorders of Very Low Birth Weight Preterms <1250 Grams. Clinical Trial Prospective, Monocentric, Randomized in Double-blinded.

Purpose

Clinical management of very low birth weight newborns (VLBW <1250g) consists in several challenges to adapt immature physiological systems to extra-uterine life. Advances in neonatal medicine for pulmonary and/or neurological and/or cardiovascular diseases have significantly improved outcomes of these children. However, the gastro-intestinal (GI) tract remains a major cause of morbidity due to

1. the immaturity of GI functions (prolonged ileus, bacterial overgrowth and translocation),
2. the complication of GI tract immaturity: intestinal perforation and enterocolitis necrotizing)
3. the need of a prolonged parenteral nutrition and its complications (central venous catheter infections, sepsis, electrolyte disturbances) but without generate a high proof level on this targeted population (<1250g).

The GI functions are progressively acquired during development and are largely sensitive to the environment, especially the intestinal luminal content. Indeed, probiotics and prebiotics have shown beneficial effects upon GI functions of newborns. One of the metabolite of the gut flora potentially involved is the butyrate. Butyrate is a short chain fatty acid produced in the colon by the microbiota (carbo-hydrates degradation). The colonic amount of butyrate increases gradually after birth. The beneficial effects of butyrate are related to its properties upon the epithelial barrier (anti-inflammatory, antioxidant, barrier repair) and upon the enteric nervous system (network of neurons and glial cells) that regulate GI functions and in particular colonic motility.

To date, there is no clinical consensus to manage digestive disorders of VLBW. Several clinical studies have assessed the effects of prokinetic drugs, dietary supplements (probiotics, prebiotics) but without generate a high proof level on this targeted population. In this context, a recent study of our Research Unit (INSERM-CIC Mère-Enfant 004) has shown benefit effects of oral probiotics supplementation in children with birth weight greater than 1000g but not in extreme preterms with birth weight less than 1000g.

The main hypothesis to explain these results lies in the intensive use of antibiotic and feeding interruption frequency in this targeted population which induce disturbances in the composition of the gut lumen (in particular the flora).

Colonic enemas assessed in various observational studies concerning VLBW seem to demonstrate a clinical efficiency upon the colonic transit, underlying by mechanical and osmotic mechanisms.

Here, the investigators propose to evaluate the clinical efficiency of butyrate enemas by a prospective randomized clinical trial blinded design.

The purpose of NEOTRANS study is to demonstrate that butyrate enemas may improve the nutritional management of extreme preterm less than 1250 grams, by facilitating the development of colic motility and clinical nutrition tolerance.

Study characteristics

Study type: interventional
Study design:
Allocation: Randomized
Endpoint classification: Safety/Efficacy Study
Intervention model: Parallel assignment
Masking: Double blind (subject/investigator)

Primary Outcome Measures:

Efficacy of colonic butyrate enemas in digestive maturation of preterms [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety: No]

The primary outcome of NEOTRANS study consists in the evaluation of the effects of colonic butyrate enemas upon the digestive maturation of preterms.

This endpoint is based on a clinical criteria that is the delay of weaning of the parenteral nutrition support. An increase of 25% (50 vs 75%) will be considered clinically significant.

Parenteral nutrition weaning is defined as the day where enteral caloric intake reach 80% of total calories.

Secondary Outcome Measures:

Gastrointestinal complications frequency [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety issue: No]

Nosocomial infections frequency [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety issue: No]

Iatrogenic effect [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety issue: No]

Whole gut transit time (red carmine test) [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety issue: No]

Growth [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety issue: No]

Comparison between 2 arms of height, weight and head circumference

Invasive ventilation support [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety issue: No]

Bronchopulmonary dysplasia incidence [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety issue: No]

Hospitalization duration [Time Frame: participants will be followed for the duration of hospital stay, an expected average of 5 weeks] [Designated as safety issue: No]

Neuromotor development [Time Frame: 2 years] [Designated as safety issue: No]

Estimated Enrollment: 136

Study Start Date: February 2012

Estimated Study Completion Date: May 2020

Estimated Primary Completion Date: May 2018
(Final data collection date for primary outcome measure)

Arms	Assigned Interventions
Experimental: Butyrate New born with a birth weight <1000g: Seven enemas of butyrate will be performed every 2 days from PND5 New born with a birth weight >1000g: Seven enemas of butyrate will be performed every day from PND5	Drug: Butyrate enemas (2.5 mM) Seven enemas Possibility to delay from 24 to 48 hours the procedure in case of clinical poor tolerance(maximum two enemas postponed and delayed at PND12 and PND13) The study remains blinded for the investigation team through the intervention of a clinical research nurse According to the procedure previously described by Nakaoka et al. (2009), a lubricated Foley catheter Ch 6 will be introduced into the rectum, the balloon will be inflated with 1 ml water for injections. Butyrate solution will be placed in a bag placed 50 cm above the child. Therefore, treatment administration will be performed at a controlled

	pressure of 50 cm H_2O without any manual intervention Installation time and retention is setted at 15 minutes Treatment units will be directly placed in the incubator 30 min before the procedure to warm the enema to +36°C Per-treatment clinical monitoring of the tolerance will be performed by a neonatologist
No Intervention: Therapeutic Abstention The protocol will pretend enema: instillation in the diaper the product under consideration, to make the two indistinguishable processes (time, odor, wicking diaper ...)	

Eligibility

Ages Eligible for Study:	up to 5 Days
Genders Eligible for Study:	Both
Accepts Healthy Volunteers:	No

Criteria

Inclusion Criteria:

- Any preterm infant with a birth weight less than or equal to 1250 grams admitted in the neonatal intensive care unit of Nantes Hospital
- Inborn or outborn
- No signs of gastrointestinal perforation or ECUN
- Absence of severe congenital disease
- Written informed consent of parental authority.

Exclusion Criteria:

- Newborn with birth weight greater than 1250 grams
- Gestational age > 30 WG
- Digestive pathology diagnosed prior PND5: perforation, necrotizing enterocolitis, malformations
- Severe congenital pathology inconsistent with clinical assessment
- Parental Refusal

Responsible Party:	Nantes University Hospital
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Thèse de Doctorat

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Ciblage nutritionnel du système nerveux entérique dans les pathologies digestives du nouveau-né prématuré

Nutritional targeting of enteric nervous system in digestive pathology of preterm newborns

Résumé

La période néonatale est une période clé dans la mise en place des fonctions digestives et dans la colonisation du tube digestif par le microbiote. Le système nerveux entérique (SNE) est un réseau de neurones et de cellules gliales situé au sein du tractus digestif qui en régule les fonctions comme la motricité et la perméabilité.

L'objectif de cette étude était de moduler le phénotype neuro-glial du SNE et/ou les fonctions digestives par des facteurs exogènes d'origine nutritionnelle.

Dans un premier temps, nous avons montré que des lavements coliques de butyrate (métabolite de la flore) chez le raton induisaient une maturation phénotypique des neurones coliques (augmentation des neurones cholinergiques et nitrergiques) associée à une augmentation de la motricité colique.

Dans un deuxième temps nous avons montré sur un modèle de co-culture que des souches bactériennes d'intérêt (bifidobactéries et lactobacilles) induisaient une modulation du phénotype neuronal de cultures primaires de SNE. La variabilité de la réponse observée était majoritairement expliquée par la souche bactérienne et non pas son espèce ou son genre.

Enfin, nous avons testé *in vivo* des souches sélectionnées par approche *in vitro* et avons montré un impact différentiel sur le phénotype neuro-glial et sur les fonctions digestives selon la souche utilisée.

En conclusion : l'utilisation de facteurs exogènes d'origine nutritionnelle en période néonatale permet d'induire une modulation des processus de maturation du SNE avec un retentissement fonctionnel conséquent permettant l'élaboration de démarches thérapeutiques innovantes chez le nouveau-né prématuré.

Mots clés :
Période néonatale, système nerveux entérique, neurone, motricité, perméabilité, butyrate, microbiote, probiotique

Abstract

The neonatal period is a key time in the development of the digestive functions and colonization of gut microbiota. The enteric nervous system (ENS) is a network of neurons and glial cells located in the digestive tract which regulates functions such as motility and permeability.

The aim of this study was to modulate the neuro-glial phenotype of ENS and/or digestive functions by exogenous factors of nutritional origin.

First, we have shown that butyrate enemas (flora metabolite) in rat pups induced a phenotypic maturation of colonic neurons (increased cholinergic and nitrergic neurons) associated with an increased colonic motility.

Secondly we have shown on a co-culture model that bacterial strains of interest (bifidobacteria and lactobacilli) induced modulations of the neuronal phenotype of primary cultures of ENS. The variability of the observed response was mainly explained by the bacterial strain and not its species or its genus.

Finally, we tested *in vivo* strains selected by the *in vitro* approach and showed a differential impact on the neuro-glial phenotype and digestive functions depending on the strain used.

In conclusion: the use of exogenous factors of nutritional origin in the neonatal period can induce a modulation of the ENS maturation process with a functional repercussion therefore allowing the development of innovative therapeutic approaches in preterm newborns.

Keywords :
Neonatal period, enteric nervous system, neuron, motility, permeability, butyrate, microbiota, probiotic