Rôle de la nutrition périnatale et du microbiote sur l'axe intestin-cerveau, la fonction endocrine intestinale et le comportement alimentaire

Gwenola Le Dréan

HABILITATION A DIRIGER DES RECHERCHES
HDR

NANTES UNIVERSITE

Spécialité : Physiologie

Par
« Gwenola CORNOU-LE DREAN »

« Rôle de la nutrition périnatale et du microbiote sur l'axe intestin-cerveau, la fonction endocrine intestinale et le comportement alimentaire »

Travaux présentés et soutenus à Nantes, le 3 octobre 2024 à 14h00
Unité de recherche : UMR 1280 PhAN, « Physiopathologie des Adaptations Nutritionnelles » INRAE-NU

Rapporteurs avant soutenance :

Pascale CHAVATTE-PALMER	Directrice de Recherche, INRAE, Jouy-en-Josas
Patricia SERRADAS	Professeure, Sorbonne Université, Paris
André BADO	Directeur de Recherche, INSERM, Université Paris Cité

Composition du Jury :

Présidente :	Khadija OUGUERRAM	Professeure, Nantes Université
Examinateurs :	Pascale CHAVATTE-PALMER	Directrice de Recherche, INRAE, Jouy-en -Josas
	Patricia SERRADAS	Professeure, Sorbonne Université, Paris
	André BADO	Directeur de Recherche, INSERM, Université Paris Cité
	Hervé BLOTTIERE	Directeur de Recherche, INRAE, Nantes Université

A Laurent Lescaudron

Remerciements

En premier lieu, je remercie vivement les rapporteurs de ce manuscrit ainsi que tous les membres de mon jury pour le temps que vous avez consacré à l'évaluation de mon parcours.

Mes remerciements vont ensuite à l'UMR PhAN qui m'accueillie en 2008 alors que j'étais encore Maître de Conférences à l'Université de Brest. J'exprime ici toute ma gratitude envers Dominique Darmaun et Martine Champ qui dirigeaient l'unité à l'époque. Leur ouverture d'esprit et leur compréhension de ma situation m'ont été d'un grand soutien.
Je remercie Jean-Pierre Segain avec qui j'ai démarré mon activité de recherche à PhAN. Il m'a offert un projet clef en main et la possibilité de co-encadrer mon premier doctorant. Je ne le remercierai jamais assez pour son soutien au moment de mon recrutement à INRAE. Je n'oublie pas non plus l'aide rassurante de Pierre de Coppet quand je suis arrivée et que je devais m'adapter au laboratoire.
Est ensuite venu le temps de la collaboration avec Patricia Parnet et Catherine Michel. Notre aventure Mamiprooffi nous a mise à rude épreuve - comment oublier les gardes de nuit à l'animalerie pour transférer nos précieux inocula de microbiote à des ratons dès la naissance- je les remercie très chaleureusement pour nos échanges constructifs mais aussi pour nos différences de point de vue qui font grandir.
Patricia, merci de ta confiance pour me transmettre la fonction d'adjointe à la direction de l'unité. Aujourd'hui je suis très heureuse de donner à PhAN en retour de son accueil il y a maintenant presque 15 ans.
Catherine, au-delà de tout ce que tu m'as apporté, merci pour ton amitié.
Je remercie très chaleureusement Marie-Cécile Alexandre-Gouabau qui m'a embarquée dans l'aventure GDM-MILK. Ta capacité à diriger cet énorme projet m'impressionne Marie-Cécile et je suis très heureuse de collaborer avec toi. Notre doctorant, Paul Bobin contribue largement à l'avancée du projet. Merci à toi Paul pour ton travail soigné et la finesse de ton humour.
Je voudrais remercier de nombreuses personnes de PhAN, qui comptent d'une manière ou d'une autre dans le quotidien de l'unité, que ce soit par l'efficacité de leur travail ou encore le lien qu'elles crée avec les autres. Certaines sont parties, Armelle, Martine, Christian, Anthony, d'autres sont encore là et j'espère pour un temps encore : Blandine, Geneviève, Alexis, Isabelle, Agnès, merci.
Je n'oublie pas les autres membres de PhAN, avec qui je partage des bons moments de travail, de convivialité et de discussions plus ou moins philosophiques selon l'humeur du moment.
Un grand merci à Hervé Blottière qui m'a poussé à soutenir l'HDR alors que j'avais renoncé.

Une pensée toute particulière et évidente pour mes trois amours, Thibault, Jeanne et Anna. Ils m'aident à avancer sans qu'ils le sachent. Des enfants devenus grands, des enfants dont le regard m'accompagne tous les jours. Merci d'être là.
Un dernier mot pour mes parents que j'ai encore la chance d'avoir, merci pour votre indéfectible soutien.

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Liste des abréviations

α-MSH: α-Melanocyte-Stimulating Hormone
α-syn : α-synucléine
5-AVAB : 5-AminoValerate Betaine
AD : maladie d'Alzheimer
ADN : Acide DéoxyriboNucléique
AGCC : Acide Gras à Chaîne Courte
AGCL : Acide Gras à Chaîne Longue
AMPK : AMP-activated protein kinase
ARN : Acide RibodésoxyNucléique
BMP : Bone Morphogenic Protein
CaMKII: Calmoduline Kinase II
CART : Cocaine- and -Amphetamine Regulated Transcript
CCK : Cholécystokinine
CCK-8S : Cholécystokine-8 sulfatée (forme active de la CCK)
CEE : Cellule Entéro-Endocrine
ChgrA ou CgA ou ChgA : Chromogranine-A
ClpB : protéine chaperonne peptidase B caséinolytique
CRCT : Congé Recherche Conversion Thématique
CTL ou CT : contrôle
DG : Diabète Gestationnel
EFS : Electric Field Stimulation
ELISA : Enzyme-Linked Immunosorbent Assay
ERK: Extracellular signal-Regulated Kinase
F-OP : Fischer-OP
F-OR : Fischer-OR
F-Sham : Fischer-Sham
FFAR-2 : Free-Fatty Acid Receptor-2
FFAR-4 : Free Fatty Acid Receptor-4
FITC : Fluoresceine IsoThioCyanate-5
GCaMP : marqueur calcique encode génétiquement (GECI), fusion de la GFP avec la calmoduline sensible au $Ca2^{+}$
GDM : Gestational Diabetes Mellitus
GFP: Green Fluorescent Protein
GHB : Gamma-HydroxyButyric acid
GIP : Glucose-dependent Insulinotropic Polypeptide
Gln ou Glc : proglucagon
GLP-1: Glucagon-Like Peptide-1
GLUTag : lignée cellulaire dérivée d'une tumeur colique de souris transgénique pour le grand antigène T sous le contrôle du promoteur du proglucagon
HFHS: High Fat High Sucrose
IMAD: Institut des Maladies de l'Appareil Digestif
KI : Knock-In
L-WRN : milieu de culture issu d'une lignée de CEE-L sécrétant Wnt3a, R spondin 3 et Noggin conditionné pour la culture de cellules souches intestinales
LP : Low Protein
MAPT : Microtubule Associated Protein Tau
MATH1: Mammalian Atonal bHLH (basic Helix-Loop-Helix) Transcription Factor 1

MCH : Melanin Concentrating Hormone
NCI-h716: lignée cellulaire dérivée d'un adénocarcinome colorectal humain
Ngn3 : Neurogenin 3
NP : Normo-Protein
NPY : Neuropeptide Y
NTS : Neurotensine
OGTT: Oral Glucose Tolerance Test
OP : Obese Prone
OR : Obese Resistant
OS: OligoSaccharides (prébiotiques)
PCNA: Proliferating Cell Nuclear Antigen
PD: maladie de Parkinson
PG : Phenylbutyrate Glutamine
PND : PostNatal Day
PYY: peptide YY
RMN : Résonance Magnétique Nucléaire
RTqPCR : Real Time (or Reverse Transcription) quantitative Polymerase Chain Reaction
SNE : Système Nerveux Entérique
STC-1 : secretin tumor cell line
TAC1 : Tachykinin precursor 1
TTX : TétrodoToxine
WT : Wild-Type

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Curriculum vitæ

Chercheure en Physiologie de l'axe intestin-cerveau et Nutrition périnatale
Directrice adjointe UMR PhAN INRAE-NU, Nantes

FORMATION SCIENTIFIQUE

1990 Licence de Biologie Cellulaire et Physiologie, Université de Rennes 1
1991 Maîtrise de Physiologie animale, Université de Rennes 1
1992 DEA de Biologie et Agronomie, « Adaptations physiologiques du jeune animal » Université de Rennes 1/AgroCampus Ouest (ex ENSAR)
1997 Doctorat de Biologie, Université de Rennes 1. Thèse en Physiologie digestive et Nutrition à l'INRA de Rennes « La sécrétion pancréatique exocrine basale et postprandiale chez le veau : implication de la cholécystokinine, de la gastrine et de leurs récepteurs » (Dir P. Guilloteau)

PARCOURS PROFESSIONNEL

1997-2000 : Post-doctorat : UMR CNRS 6026 **Interactions cellulaires et moléculaires**, équipe « Endocrinologie Moléculaire de la Reproduction »**,** Université de Rennes1 (O. Kah). « Influence de la photopériode (mélatonine) sur la fonction gonadotrope chez la truite et recherche d'une horloge centrale endogène »

2000-2009 : Maître de conférences Section CNU 66, Physiologie : Université de Brest**,** Ecole Supérieure de Microbiologie et Sécurité Alimentaire de Brest (actuelle ESIAB)

❖ **Enseignement** (> 192h TD/an) :

- Responsable de l'UE Qualité des Productions Animales (84 h): conception et mise en œuvre de l'UE, gestion des intervenants extérieurs, présentation des filières, visites d'élevages, interprofession.
- TP de Biochimie (60 h) et de Biologie moléculaire (40 h), mise en place de nouveaux TP
- Responsable de l'UE de Nutrition Humaine (26 h)
- Suivi des stages, rapports bibliographiques et projets d'étude

❖ **Recherche**

Laboratoire de Microbiologie et Sécurité Alimentaire (ERT 16, Pr Y. Tirilly), équipe de Toxicologie Alimentaire (2001-2003)

- Hémato-toxicologie : effets des mycotoxines sur progéniteurs hématopoïétiques humains et murins ; apoptose
- Immuno-toxicologie des cellules dendritiques

Laboratoire de Biodiversité et Ecologie Microbienne (EA 3882, Pr G. Barbier) (2004-2008)

- Développement de l'axe physiologie du laboratoire : recherche de marqueurs de stress fongique

2009-2010 : Congé recherche et conversion thématique (CRCT, CNU 66) dans l'UMR (Dir. Pr D. Darmaun) avec mobilité géographique. Equipe « Adaptation nutritionnelle de la muqueuse colique » (Dr J. P. Segain) : Rôle du tissu adipeux viscéral sur l'intégrité de la muqueuse colique dans un modèle de retard de croissance intra-utérin

Depuis 2010 : Chargée de recherche dans l'UMR PhAN INRAE-NU (Dir. Dr H. Blottière depuis 2022). Effet de la dénutrition périnatale sur le fonctionnement de l'axe intestin-cerveau : cellules entéro-endocrines, nerf vague et régulation de la prise alimentaire

Financements et Coordination de projets

2005-2007 : Partenariat avec Lactalis : quantification par PCR quantitative de flore fongique technologique sur fromages au cours de leur maturation. Transfert de méthodologie auprès des techniciens Lactalis.

2014-2017 : Co-porteur du projet scientifique PARIMAD « Impact de la dénutrition périnatale sur le système neuroendocrine intestinal » financé par la Région des pays de La Loire. Subvention pour une allocation doctorale sur la période.

2015-2016 : Partenariat industriel avec Olygose « Effets d'une supplémentation néonatale en oligosaccharides prébiotiques sur le connectome intestinal et le comportement alimentaire adulte »

2017-2020: ANR Mamiprooffi « Impact of maternal-to-pup vertical transfer of obesity-associated microbiotas on early neurogenesis and development of neuronal circuitries regulating eating behavior in rat offspring » (P. Parnet)

2020-2021 : subventions fondation Santédige, CHU Nantes

2013 & 2019, 2020, 2021, 2022 : Subventions ANSSD allouées par le département ALIMH d'INRAE

2022: ANR GDM-Milk « Breast milk as a new paradigm for preserving offspring born from gestational diabetes-mother against adult diabetes » (projet co-écrit avec et porté par MC. Alexandre-Gouabau)

2023: ANR EnteroendoPark « Enteroendocrine cells, tau and alpha-synuclein, partners in crime in Parkinson's disease » (projet co-écrit avec et porté par P. Derkinderen)

Co-encadrements de thèses

HAURE MIRANDE Vianney (2009-2013) : « Mécanismes épigénétiques et métaboliques impliqués dans la programmation fœtale des pathologies coliques ». Soutenue le 7 juin 2013 à l'Université de Nantes. Directeur : Jean-Pierre Segain. Co-encadrement à 50%. Actuellement chercheur à l'Icahn School of Medicine, Departments of Neurology and Pediatrics, Mount Sinai, New York, USA.

Publications associées : #6 et #9 ; Communications associées : #PO56, #PO54, #PO53, #P52, #P47, #PO46, #P45, #P43, #P42, #P41, #PO40, #P39, #PO30

NDJIM Marième (2014-2017) : « Impact de la dénutrition périnatale sur le système neuroendocrine intestinal ». Soutenue le 14 décembre 2017 à l'Université de Nantes. Directeur : Michel Neunlist. Co-encadrement à 60%. Actuellement Business Developer chez Genoway (Lyon).

Publications associées : #5 et #7 ; Communications associées : #PO26, #P25, #P22, #P20, #P19, #P18

POCHERON Anne-Lise (2015-2019) : « Rôle des microbiotes maternels dans le développement précoce des réseaux cérébraux régulant le comportement alimentaire du jeune rat, dans un contexte d'obésité maternelle ». Soutenue le 18 décembre 2020 à l'Université de Nantes. Directrice : Patricia Parnet. Co-encadrement à 50%. Actuellement Business Developer chez Biofortis (Saint-Herblain)

Publications associées : #3, #4 et #32 ; Communications associées : #PO17, #PO16, #P15, #P14, #PO13, #PO12, #P9, #P7, #P6, #PO4

BOBIN Paul (Nov 2021-) : « La lactation comme période de reprogrammation du système métabolique et endocrine de la descendance exposée *in utero* à l'hyperglycémie maternelle ». Directrice : Marie-Cécile Alexandre-Gouabau. Co-encadrement à 40%.

Communications associées : #P5, #PO4, #P3, #PO1

Encadrements et Co-encadrements de Master 2

LE CALVEZ Thomas (2005) : Master Microbiologie fondamentale et appliquée, Université de Brest

FONDREVEZ Marc (2006) : Développement d'une méthode de quantification par PCR en temps réel du genre *Penicillium* dans les fromages : étude préliminaire. Master pro Maîtrise et Utilisation des Micro-organismes, Agrocampus Ouest

HABRYLO Olivier (2007) : Essais de quantification de *Penicillium camemberti* et *Penicillium roqueforti* en matrice fromagère par PCR en temps réel. Master Microbiologie fondamentale et appliquée, Université de Brest. **Publication associée #13.**

POINSIGNON Camille (2014) : Empreinte nutritionnelle et dérégulation de la prise alimentaire : étude de la sensibilité à la cholécystokinine chez le rat. Master Science de l'Aliment et Nutrition Humaine, Université de Nantes. **Publication associée #7. Communications associées #PO34 et #P36**

OLIER Astrid (2015) : La maturation néonatale des cellules entéro-endocrines est-elle affectée par une supplémentation en prébiotique, chez le rat ? Master Biologie Santé, spécialité BBRT (Biologie Biotechnologie et Recherche Thérapeutique), Université de Nantes. **Communication associée #P33, #P32, #P29, #P24**

GOUYON Edith (2016) : Effet d'une supplémentation néonatale en oligosides prébiotiques sur la maturation du système nerveux entérique chez le rat. Master Sécurité de l'Aliment et Nutrition Humaine, Université de Nantes, **Communication associée #P27**

HOUSSAIS Bastien (2018) : Caractérisation fonctionnelle et *in vitro* du microbiote intestinal dans un modèle de rat prédisposé à l'obésité. Master Biologie Santé, spécialité BBRT (Biologie Biotechnologie et Recherche Thérapeutique), Université de Nantes. **Communication associée #PO4**

QUEIGNEC Maiwenn (2020) : Effets à court et long terme d'une supplémentation orale de miRNA à des ratons nouveau-nés sur les cellules endocrines intestinales. Master Biologie Santé, parcours Nutrition Santé Humaine, Université Clermont Auvergne. **Publication associée #2. Communications associées #P10, #P11**

MOUSSAVI SERESHT Seyed Kourosh (2021) Impact d'un Transfert de Microbiotes Maternels sur le Transcriptome Cérébral et Intestinal de Jeunes Ratons. Master Nutrition et Sciences des Aliments, Université de Nantes-Agrocampus Ouest-Université de Rennes1

MICHELLAND Marie (2023) Impact de composés bioactifs du lait maternel de femmes en situation de diabète gestationnel sur la fonction incrétine de la descendance. Master PREVALS, Alimentation et Santé, cycle ingénieur, UniLaSalle Beauvais

BARD Camille (2023) : Le microbiote transmis par la mère peut-il influencer le risque de développement des troubles du spectre autistique et des troubles du déficit de l'attention avec hyperactivité ? Synthèse bibliographique en Biologie, Master Biologie-Gestion, Université de Rennes.

MASSIAS Corentin (2024) Effets du lait maternel sur la maturation postnatale de la fonction endocrine pancréatique et intestinale dans un contexte de diabète gestationnel. Master Biologie Santé, spécialité BBRT (Biologie Biotechnologie et Recherche Thérapeutique), Nantes Université

Encadrement Master 1 et TER

MAHE Aurélie (2013) : Rôle de la cholécystokinine sur la plasticité neurochimique vagale dans un modèle de programmation métabolique. Master Biologie Santé TER (parcours Sciences Biologiques), Université de Nantes

BERTHONNET Adrien (2013) : La différenciation des cellules entéro-endocrines, clé dans la plasticité de réponses de l'intestin. Rapport bibliographique, Master Sciences des Aliments et Nutrition Humaine, Université de Nantes.

REUFFLET Aurélie (2016) : Effets d'une supplémentation néonatale en oligosaccharides prébiotiques, à court et long termes, sur le phénotype des cellules entéro-endocrines et le comportement alimentaire chez le rat. Master Biologie Santé TER (parcours Santé), Université de Nantes

MERIAUX Rose (2016) : Effet d'un retard de croissance intra-utérin sur la prolifération et la différenciation des cellules entéro-endocrines chez le rat. Master Biologie Santé TER (parcours Sciences Biologiques), Université de Nantes

LECERF Chloé (2017) : Impact d'une restriction protéique périnatale sur la prolifération, la différenciation et la maturation des cellules entéro-endocrine chez le rat. Master Biologie Santé TER (parcours Sciences Biologiques), Université de Nantes

PHILIPE Lou (2019) Effet d'un retard de croissance intra-utérin sur la prolifération et la différenciation des cellules entéro-endocrines chez le rat GFP. Master Biologie Santé TER (parcours Santé), Université de Nantes

TRUIN Tanguy (2019) : Effets *in vitro* des eaux caecales de ratons prédisposés ou résistants à l'obésité par transfert des microbiotes maternels sur des lignées de cellules entéro-endocrines. Master Biologie Santé TER (parcours Sciences Biologiques), Université de Nantes

GARCIA Adrien (2021) : Diabète gestationnel, allaitement et prédisposition au syndrome métabolique de la descendance. Master Biologie Santé TER bibliographique (parcours Sciences et Santé), Université de Nantes

MADEC Apolline (2021) : La nutrition périnatale a-t-elle un impact à long terme sur les cellules entéro-endocrines productrices d'incrétines ? Master Biologie Santé TER bibliographique (parcours Sciences et Santé), Université de Nantes

EVANNO Lisa (2022) : Expression de la protéine Tau dans l'épithélium intestinal en conditions inflammatoires. Licence 3 SV, mention ABT, UFR Sciences et Techniques, Nantes Université

De BALANDA Audrey (2022) : Mise en place d'un modèle rongeur de diabète gestationnel. Master Biologie Santé TER (parcours Sciences et Santé), Nantes Université

CLEUZIOU Léa (2023) : Expression de la protéine Tau dans les cellules entéro-endocrines coliques. Licence 3 SV, mention ABT, UFR Sciences et Techniques, Nantes Université

LEGRAND Marie (2023) : Impact du diabète gestationnel et de l'allaitement sur le développement de troubles de type TSA et TDAH chez la descendance : exploration comportementale dans un modèle murin. Master Nutrition et Sciences des Aliments, Université de Bordeaux.

Encadrement Formations courtes

DURAND Pauline (2011) Empreinte nutritionnelle et fonction endocrine intestinale. Brevet Technicien Supérieur en Biotechnologies, Lycée Talensac, Nantes

BEGHIN Agnès (2017) Effets à long terme d'une supplémentation néonatale en prébiotiques sur les cellules entéro-endocrines chez le rat. Brevet Technicien Supérieur en Biotechnologies, Lycée Talensac, Nantes

Formations à l'encadrement doctoral et au management

2015 : Séminaire Encadrants (2j) « Encadrement du projet doctoral » animé par Asceo pour INRAE

2016 : EDEN, Ecole des Doctorants et des Encadrants d'INRAE (4j en immersion avec le doctorant) animée par Asceo, à Ecully (38)

Depuis 2020 : membre du groupe ALBEDO « Accueil du binôme Encadrant-Doctorant » porté par le Centre INRAE des Pays de La Loire, 1 animation annuelle sur les 3 ans de doctorat. Montage et animation des ateliers (Alignement des attentes, Montée en compétences du doctorant, Ethique en recherche).

2021-2023 : cycles de formation (7 journées) à l'encadrement de proximité INRAE (Cabinet Cohérences pour INRAE)
2023 : management hybride (12h en distanciel, Cabinet Cohérences pour INRAE)
2024 : conduite d'entretiens individuels (groupe des directeurs d'unités INRAE, 6h)

Comité de suivi individuel (depuis 2014)

Mathilde Guerville, NUMECAN, INRAE, Rennes
Thomas Clairembault, TENS, INSERM-NU, Nantes
Chloé Lefèvre, TENS, INSERM-NU, Nantes
Damien Garçon, ITX, INSERM-NU, Nantes
Adrien de Guilhem de Lataillade, TENS, INSERM-NU, Nantes
Morgane Le Dréan, Tens, INSERM-NU, Nantes
Laudy Serhal, BIOEPAR, INRAE, Nantes

Participation jury de thèse

2013 : V. Haure-Mirande (co-encadrante)

2017 : M. Ndjim (co-encadrante)

2018 : X. Zhang, INRAE Micalis Jouy-en-Josas (examinatrice)

2019 : A.L. Pocheron (co-encadrante)

Organisation et animation de séminaires

Depuis 2017 : Journées Microbiotes et Santé, Séminaires destinés aux étudiants de master 2 (BBRT & RC, option PACI & MI, NSA) ouverts aux cliniciens et scientifiques. Devient en 2024 la Winter School Cursus Master Doctorat (Graduate Grogram) MICAS (Microbiote-Intestin-cerveau-Alimentation-Santé)

Depuis 2013 : Journée annuelle scientifique de l'IMAD destinée aux jeunes chercheurs des unités (principalement aux M2)

Invitations à des colloques

Congrès de clôture du consortium Mibiogate, Session « L'axe Microbiote-Intestin-Cerveau dans les troubles du comportement alimentaire », 11-12 mars 2022, Nantes

Journées Francophones de Nutrition, Session « Risque métabolique de l'enfant à l'adulte », 6-8 décembre 2023, Marseille

Responsabilités/ Membre de conseils /Jury de Master

Actuellement :

- Directrice adjointe de l'UMR (depuis mars 2023)
- Conseils scientifiques : SFR F. Bonamy; CS Pôle Santé; Comité de Pilotage de l'EUR Biologie-Santé (Graduate School)
- Equipe pédagogique et comité de pilotage du Cursus Master Doctorat (Graduate Program) MICAS
- Directoire de l'Institut des Maladies de l'Appareil Digestif, CHU Nantes : responsable des actions de formation et d'animation scientifique
- Bureau du CECED (Club d'Etudes des Cellules Epithéliales Digestives)
- Commission de préparation au Jury d'examen de master2 BBRT NU
- Jury de soutenances Master2 BBRT (depuis 2013)

Précédemment :

- Jury de Master2 BBRT NU (2013-2017)
- Représentante de l'UMR PhAN à l'Ecole Doctorale « Biologie-Santé » de l'Université de Nantes (2010-2016).
- Participation à l'organisation des Doctoriales du PRES LUNAM (Mars 2014)
- CNU 66ème section (Physiologie) : membre nommé 2004-2007; 2012-2015 ; 2016-2019
- Membre de jury de recrutement au concours de l'Ecole Doctorale (2012)
- Membre de comités de sélection (ONIRIS, IUT Strasbourg) concours MCU
- 2004 - 2008 : membre élue de la CSE 66ème section (Université de Brest)
- 2002-2007 : membre élue du conseil d'administration de l'ESMISAB
- Jury de recrutement des étudiants, suivi de leur insertion professionnelle (enquêtes)
- Responsable Référentiel CDEFI (Conférence des Directeurs des Ecoles Françaises d'Ingénieurs) pour l'ESMISAB

Reviewings récents

Nutrients (4), Foods (1), Microrganisms (1), J Clin Nutr and Food (1), British Journal of Nutrition (1), Scientific Reports (1), Frontiers in neuroscience (1)

Synthèse des travaux 1998-2023

Avant-propos

Depuis l'obtention de mon doctorat en 1997, la trajectoire de ma carrière scientifique s'est trouvée à plusieurs reprises déviée par des éléments de contextes familiaux et professionnels me conduisant à opérer des changements thématiques majeurs. Les adaptations que nécessitent ces transitions impactent forcément sur l'acquisition de l'expertise scientifique dans un domaine en particulier et la reconnaissance qui en découle mais permettent un enrichissement personnel très fort nourri par la curiosité scientifique. L'ensemble des travaux réalisés depuis la thèse a construit ma vision de la physiologie intégrative que je tente de transmettre aux étudiants que j'encadre.

La frise ci-dessous représente mon parcours professionnel depuis le doctorat.

Synthèse des travaux 1998-2009

I. POST-DOCTORAT (1998-2000) : NEUROBIOLOGIE DE LA REPRODUCTION

Laboratoire d'Endocrinologie Moléculaire de la Reproduction UPRES-A CNRS 6026 à l'Université de Rennes 1 (O. Kah)
Projet européen (FAIR PL. 961410) « Environmental and Neuroendocrine control mechanisms in finfish reproduction and their application in broodstock management ».

1. OBJET ET FINALITÉS

Un des objectifs était d'étudier la régulation centrale de la photopériode sur le fonctionnement de l'axe gonadotrope et des rythmes circadiens chez la truite. Outre une meilleure compréhension des mécanismes d'intégration du signal mélatonine, la finalité de l'étude consistait à améliorer les pratiques photopériodiques qui provoquent souvent des effets indésirables sur la qualité des pontes et la survie larvaire.

2. INTÉRÊT SCIENTIFIQUE

Cette équipe s'intéressait à l'influence de facteurs environnementaux (variations de la photopériode, stress, xénobiotiques) sur le fonctionnement de l'axe gonadotrope. Chez la plupart des animaux à période de reproduction saisonnière (Ovins, Hamster, Poissons, etc.), la photopériode, c'est-à-dire la variation annuelle de la durée de la phase d'éclairement, détermine l'entrée en période de reproduction. La production de mélatonine est gouvernée par l'alternance jour/nuit et régule l'activité des rythmes endogènes eux-mêmes sous le contrôle d'une horloge interne. L'existence d'une telle horloge interne chez la truite n'était pas clairement établie.

3. PROGRAMME DE TRAVAIL ET MÉTHODES

Les effets de la photopériode (mélatonine) ont été étudiés sur l'expression du récepteur aux œstrogènes (ER), impliqué dans le rétrocontrôle œstrogénique sur la sécrétion des hormones gonadotropes *in vitro* et *in vivo*. *In vitro*, nous avons utilisé un système de culture d'hépatocytes en agrégats. En effet, la surexpression d'ER et de la vitellogénine en présence d'œstradiol dans le foie de truite permet de quantifier aisément (dot blots) les variations d'expression de ces 2 gènes. Une étude *in vivo* a été menée en parallèle chez des poissons ayant reçu des implants de mélatonine ou ayant été pinéalectomisés.

Une cartographie de l'expression des récepteurs à la mélatonine et de *clock*, gène intervenant dans le fonctionnement des rythmes circadiens, a été réalisée dans le cerveau par hybridation *in situ*. La mise au point de la technique de protection à la RNAse (RPA) devait permettre l'analyse des effets de la photopériode au niveau central sur l'expression de ER, du récepteur à la mélatonine ainsi que celle de *clock*.

4. RÉSULTATS

L'hybridation *in situ* dans le cerveau a montré que le récepteur à la mélatonine est exprimé dans les systèmes visuels primaires incluant le thalamus, l'aire prétectale, les toits optiques et le cervelet. Une expression moins importante apparaît dans d'autres régions du cerveau, notamment dans l'aire préoptique et l'hypothalamus médiobasal. Enfin, l'hypophyse exprime très faiblement le récepteur à la mélatonine. La mélatonine n'a pas d'effet sur l'expression stimulée par l'œstradiol d'ER et de la vitellogénine *in vitro* et aucune interaction mélatonine/ER n'a été observée *in vivo* (publication #18, Mazurais *et al.*, 2000).

Ces résultats montrent à la fois l'expression du récepteur à la mélatonine et la présence de sites de liaison fonctionnels dans les régions impliquant les voies visuelles primaires. Les résultats d'hybridation *in situ* ont montré la co-expression du gène *clock* et du récepteur à la mélatonine dans les toits optiques et l'aire prétectale (publication#19, Mazurais *et al.*, 2000). Ceci suggère l'existence d'organisations cellulaires pouvant jouer le rôle d'horloge interne dans le système nerveux central de truite arc-en-ciel. La suite de ce travail consistait à établir les modalités de l'expression jour/nuit du récepteur à la mélatonine et de *clock* dans le cerveau,

notamment dans les toits optiques. Malheureusement, la lourdeur de la technique de RPA n'a pas permis d'approfondir cet aspect de façon satisfaisante.

En septembre 2000, j'ai été recrutée en qualité de Maître de conférences (section CNU 66, Physiologie) à l'ESMISAB (Ecole Supérieure de Microbiologie et Sécurité des Aliments de Brest, actuelle ESIAB) pour y enseigner la Qualité des Productions Animales. Mon travail de recherche s'est déroulé dans 2 équipes différentes entre 2001 et 2007.

II. PARCOURS ENSEIGNANT-CHERCHEUR (Université de Brest)

A. Thématique « Toxicologie alimentaire cellulaire » (2001-2003)

Laboratoire de Microbiologie et Sécurité Alimentaire (ERT 16, Y. Tirilly), Université de Brest, ESMISAB.

L'équipe de Toxicologie était spécialisée dans la mise au point de tests permettant d'évaluer l'effet de mycotoxines au cours de l'hématopoïèse humaine et murine, notamment sur les cellules de la lignée myéloïde produisant les globules blancs, les globules rouges et les plaquettes.

1. OBJET ET FINALITÉS

L'étude qui m'a été confiée consistait à évaluer la myélotoxicité des trichothécènes sur les progéniteurs hématopoïétiques précoces humains $CD34^+$ (non déterminés vers la lignée lymphoïde ou myéloïde).

2. INTÉRÊT SCIENTIFIQUE

Les trichothécènes (180 identifiés) sont des mycotoxines produites par différentes espèces de *Fusarium*, champignons qui se développent notamment sur les grains de céréales destinés à l'alimentation humaine et animale. La toxine T-2 est la plus toxique connue à ce jour et le déoxynivalénol (DON) la plus répandue. Les trichothécènes sont à l'origine de troubles hématologiques (leucopénie, thrombocytopénie, etc.) et immunologiques majeurs. Ce sont des inhibiteurs puissants sur la synthèse des protéines dans les cellules eucaryotes via un stress ribotoxique induisant l'apoptose. Ce mécanisme n'avait pas été encore exploré pour expliquer leur myélotoxicité.

3. PROGRAMME DE TRAVAIL ET MÉTHODES

Extraites de sang de cordon ombilical humain à partir de placentas issus d'accouchements normaux, les progéniteurs hématopoïétiques $CD34^+$ sont purifiés par sélection négative à partir des cellules mononucléées. Après prolifération *in vitro*, les cellules $CD34^+$ étaient incubées en présence de toxine T-2 ou de DON (10^{-7} à 10^{-10} M) pendant 2 à 24h.
L'apoptose a été mesurée par coloration de Hoechst, fragmentation de l'ADN, perte d'asymétrie membranaire (marquage annexine-V/iodure de propidium en cytométrie de flux). Des études clonogénique ont été réalisées pour déterminer si les effets des trichothécènes sur les progéniteurs influençaient la prolifération et l'engagement de ces derniers dans l'une ou l'autre des lignées.

4. RÉSULTATS

L'induction de l'apoptose par la toxine T-2 mais pas par le déoxynivalénol a été montrée *in vitro* sur les progéniteurs $CD34^+$. L'induction d'apoptose par la toxine T-2 est dose et temps-dépendante. L'inhibition de ce phénomène par l'ajout d'un inhibiteur de caspases (Z-VAD-fmk) et la mesure de l'activité de la caspase-3 ont montré l'implication de cette enzyme dans la réponse apoptotique. Les études clonogéniques réalisées sur les progéniteurs $CD34^+$ après induction d'apoptose par la toxine T-2 montrent une forte inhibition de la prolifération des

cellules. Ceci suggère que la myélotoxicité de la toxine T-2 pourrait s'expliquer au moins en partie par une induction d'apoptose. Les lignées de progéniteurs hématopoïétiques montrent des sensibilités différentes au DON. La cytotoxicité de cette mycotoxine a été observée uniquement sur la lignée granulo-macrocytaire et les résultats obtenus dans la présente étude montrent que le mécanisme n'est pas apoptotique (publication#15, Le Dréan *et al.*, 2005). *Ces travaux ont permis d'apporter des données mécanistiques sur la myélotoxicité des trichothécènes et de développer de nouveaux outils d'évaluation toxicologique. J'ai participé également à l'étude de la toxicité des trichothécènes sur cellules circulantes* (publication#16, Froquet *et al.*, 2003) *montrant que celles-ci étaient peu affectées par les mycotoxines, renforçant ainsi notre intérêt pour les études sur les progéniteurs.*

Afin de compléter la batterie de tests permettant d'évaluer la toxicité des mycotoxines *in vitro*, il m'avait été demandé de mettre en place la culture primaire de cellules dendritiques. Les progéniteurs $CD34^+$, sous l'action de cytokines appropriées, prolifèrent et se différencient en cellules dendritiques dont le phénotype correspond à celui de celles localisées dans la plupart des épithéliums de l'organisme (peau, intestin, poumons, etc.). Ce modèle a servi à l'étude des effets immuno-toxiques des mycotoxines dans le cadre de la **thèse de N. Hymery que j'avais commencé à encadrer.** *Pour différentes raisons, cette équipe a quitté l'ESMISAB début 2004 et je n'ai pas pu poursuivre ces travaux. J'ai donc intégré le laboratoire de microbiologie de l'ESMISAB.*

B. Thématique « Microbiologie alimentaire » (2004-2007)

Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (EA 3882, G. Barbier)
Université de Brest, ESMISAB
Projet industriel Lactalis (Retiers, 35) : quantification de la biomasse fongique d'affinage dans les matrices fromagères

1. OBJET ET FINALITÉS

J'avais la responsabilité d'un projet en collaboration avec le Laboratoire de Recherche et Développement de Lactalis visant à mettre au point une méthode de quantification absolue de la biomasse fongique d'intérêt technologique dans les fromages**. J'ai encadré 2 étudiants de master2 dans ce projet (Marc Fondrevez et Olivier Habrylo).**

2. INTÉRÊT SCIENTIFIQUE

Le suivi de la flore technologique dans la production et la maturation des fromages est un élément important permettant de contrôler l'implantation de cette flore positive et d'assurer une meilleure maîtrise des processus. *Penicillium camemberti* et *P. roqueforti* sont deux champignons filamenteux très largement utilisés dans la production de fromages traditionnels et industriels. La dynamique de croissance de ces deux espèces dans les fromages est généralement suivie à l'aide de méthodes culturales sur milieu gélosé. Cette technique permet la numération des spores présentes dans l'échantillon mais ne reflète aucunement le recouvrement mycélien qui a lieu au cours de la maturation des fromages.

La PCR quantitative est couramment utilisée pour quantifier spécifiquement une population de bactéries ou les spores de champignons car il est aisé d'extrapoler la quantité d'ADN à un nombre de cellules. Dans le cas du mycélium, constitué d'hyphes plurinucléés, cette extrapolation n'est pas applicable directement. L'originalité de ce travail a consisté à mettre au point une méthode permettant d'appliquer la qPCR à du mycélium, sur matrices fromagères.

3. PROGRAMME DE TRAVAIL ET MÉTHODES

La première étape de ce travail a consisté à montrer que l'ADN constitue un marqueur pertinent de la croissance mycélienne de *P. camemberti* et *P. roqueforti*, d'abord en cultures pures liquides. Un protocole d'extraction d'ADN à partir de mycélium a été mis en place.

L'ADN extrait était dans un premier temps quantifié sur gel d'agarose pour mesurer le ratio ADN extrait / masse de mycélium au cours du temps et en fonction de la température. Puis, un protocole de qPCR en temps réel sur cultures pures, pour chaque espèce, a été mis au point.

L'étape suivante a consisté à valider l'approche expérimentale sur matrices fromagères. L'échantillonnage, la solubilisation et la délipidation de celles-ci ont été optimisées. Afin de répondre aux exigences des industriels, le protocole de qPCR a ensuite été adapté de façon à transformer directement la fluorescence en biomasse fongique. Pour cela, nous avons réalisé des standards contenant des quantités connues de mycélium frais pour 1 g de matrice et vérifié la linéarité des rendements d'extraction. Les gammes de qPCR ont été construites à partir de dilutions successives d'un de ces standards, ce qui a permis d'exprimer les résultats non plus en quantité d'ADN comme cela est classiquement le cas mais directement en concentration de mycélium dans la matrice.

4. RÉSULTATS

Sur culture pure en milieu liquide agité, nous avons montré que le rapport entre la quantité d'ADN extraite et la biomasse sèche de départ (entre 0 et 20 mg) était constant au cours de la croissance des 2 espèces de *Penicillium*. Les résultats de qPCR obtenus à partir des standards montrent que les quantités d'ADN récupérées sont proportionnelles à la quantité de mycélium de départ. Ces résultats ont permis de valider l'ADN comme marqueur de biomasse dans les matrices ainsi que le protocole de solubilisation et de délipidation de la matrice.

L'application de la méthode sur des échantillons de fromages en cours d'affinage a permis d'apporter un nouvel éclairage sur le développement de la flore d'affinage fongique. La croissance mycélienne est exponentielle dès les premiers jours qui suivent l'ensemencement. Mesurée avec les méthodes culturales classiques, cette phase de croissance n'est pas détectée (publication#13, Le Dréan *et al.*, 2010**, O. Habrylo M2 associé**).

La méthode mise au point a fait l'objet d'un transfert vers la société Lactalis et d'une formation auprès des techniciens de leur laboratoire R&D. Pour la première fois, les fromagers industriels disposent d'un outil leur permettant de suivre quantitativement le développement de la biomasse fongique sur leurs produits. Les données qu'ils vont pouvoir obtenir à l'aide cet outil leur seront essentielles pour mieux comprendre les modifications physico-chimiques qui ont lieu au cours de la maturation des fromages et ainsi de mieux en maîtriser la qualité organoleptique (publication#33, Le Dréan, 2010).

Dans cette équipe, j'ai participé à une étude transcriptomique pour rechercher marqueurs de stress chez *Penicillium glabrum*, moisissure contaminante de produits agroalimentaires. Ma contribution à ce travail a consisté à mettre en place la PCR en temps réel pour valider les résultats de puces à ADN, par la recherche et la validation d'invariants permettant de mesurer l'expression relative de certains gènes lors d'un stress thermique (publication#14, Névarez *et al.*, 2008).

Synthèse des travaux depuis 2009

En 2007, dans le cadre d'une mobilité liée à un rapprochement familial à Nantes, j'ai continué à assurer mon service d'enseignement à Brest et intégré l'UMR PhAN à Nantes. Le choix de cette unité me permettait de revenir vers ma formation initiale en physiologie digestive et nutrition. En 2009, j'ai obtenu un Congé Recherche et Conversion Thématique (CRCT) d'un an au titre de la Commission Nationale Universitaire (66ème section). En 2010, j'ai obtenu par concours externe un poste de Chargé de Recherche à l'UMR PhAN.

III. PARCOURS CHERCHEUR « NUTRITION PÉRINATALE ET AXE INTESTIN-CERVEAU » à l'UMR PhAN (depuis 2009)

A l'UMR PhAN, mon activité de recherche s'inscrit dans la thématique de l'origine développementale de la santé et des maladies (DOHaD) et des 1000 premiers jours (**Figure 1**). Ce concept, issu des études épidémiologiques de Barker dans les années 90, veut qu'un environnement fœtal altéré, en cas de malnutrition maternelle par exemple, induise une programmation* des organes vers un phénotype économe (Hales *et* Barker, 2001). Face à un environnement différent au cours du développement ou plus tard dans la vie (mismatch), ce phénotype serait plus difficilement capable de s'adapter et serait à l'origine de susceptibilités accrues aux pathologies à l'âge adulte et notamment les maladies métaboliques (diabètes, dyslipidémies, obésité) (Gluckman *et al.*, 2018).

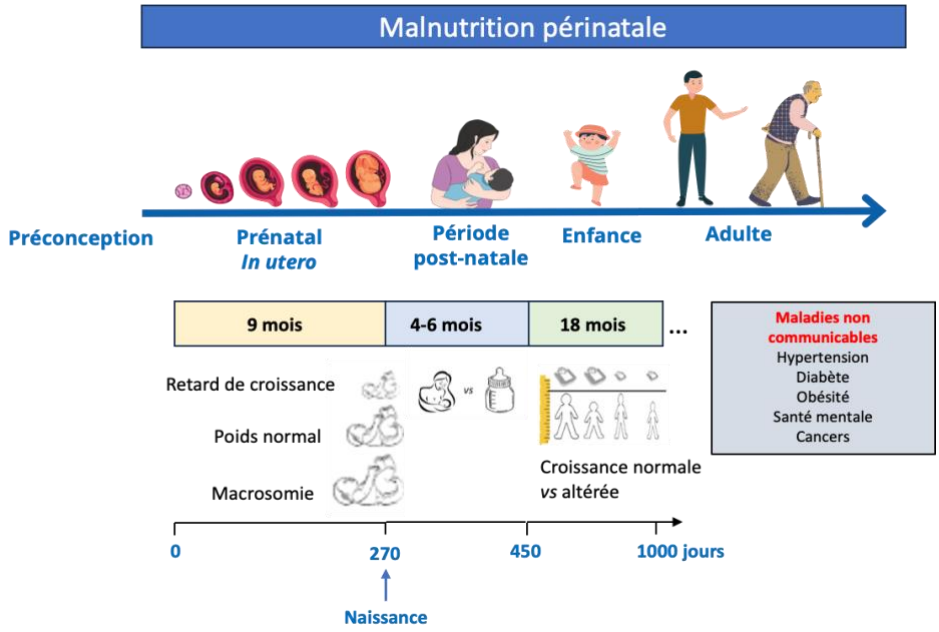


Figure 1. Le concept de la DOHaD illustré par les conséquences d'une malnutrition périnatale sur le risque de maladies chroniques plus tard dans la vie. La grande plasticité développementale durant les 1000 premiers jours de vie fait de cette période une fenêtre critique de sensibilité à l'environnement.

*Programmation /Empreinte : quelle définition ?

Le terme « programmation » renvoie aux effets à long-terme, à distance d'évènements précoces. Le concept de la DOHaD repose sur le fait qu'une empreinte environnementale, nutritionnelle ou métabolique est déposée sur les organes aux stades précoces du développement. Cette empreinte constitue un ensemble de facteurs de risque de développer des maladies plus tard dans la vie. Les marques de cette empreinte sont complexes, elles peuvent être épigénétiques mais aussi altérer la structuration et le fonctionnement des organes dont l'impact ne sera visible qu'à long-terme, en fonction de l'exposition à d'autres stresseurs. Ainsi le terme « programmation » qui sous-tend le plus souvent des mécanismes prédéfinis, orchestrés et régulés comme ceux qui se déroulent pendant le développement embryonnaire n'est pas complètement adapté aux effets potentiels et parfois réversibles de la nutrition en début de vie sur la santé future. Il est néanmoins souvent utilisé de même que le terme « empreinte » dans le contexte de la DOHaD.

Compte tenu de la transmission intergénérationnelle de ce risque (revue Patti, 2013), la DOHaD est un enjeu de santé publique majeur nécessitant la mise en place de stratégies d'interventions périnatales, notamment nutritionnelles (**Figure 2**) (Revue Nemoto *et* Sagawa, 2023).

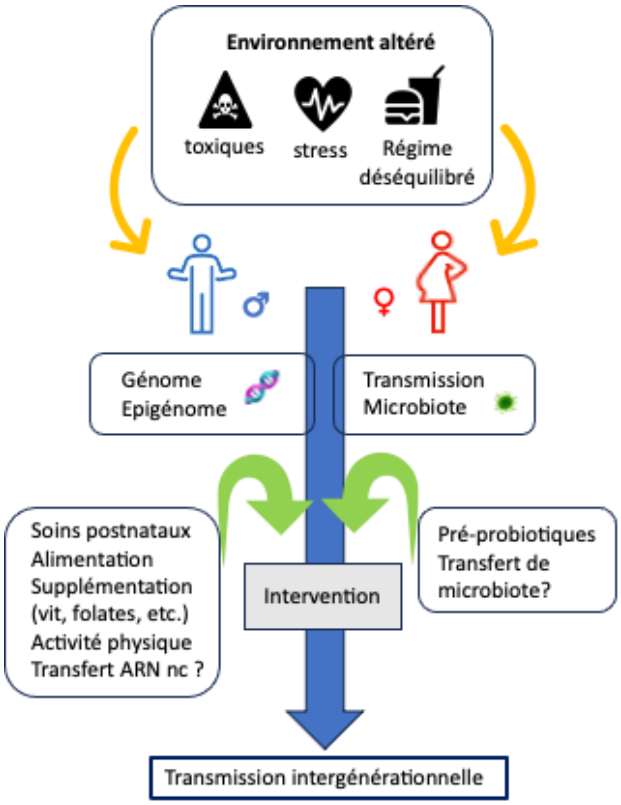


Figure 2. Transmission parentale des altérations environnementales à la descendance et stratégies d'intervention. (Adapté de Nemoto *et* Sagawa, 2023). Les mécanismes impliqués à la transmission au fil des générations du risque de maladies chroniques, pourraient être soit associés au génome (marques épigénétiques : acétylations d'histones, méthylations de l'ADN, ARN non codants) ou non (microbiome). L'efficacité des interventions proposées reste à démontrer pour la plupart chez l'homme. Vit, vitamines ; ARNnc, ARN non codants.

Le modèle de programmation nutritionnelle/métabolique développé à l'UMR Phan au moment de mon arrivée était le rat né avec un retard de croissance intra-utérin (RCIU) obtenu par restriction protéique des rates gestantes et/ou allaitantes. En effet, la majorité des études humaines utilisent le petit poids de naissance (<2,5 kg) ou l'insuffisance pondérale pour l'âge gestationnel comme indicateur d'un développement intra-utérin sous-optimal. Une insuffisance placentaire, un apport énergétique et/ou nutritionnel inadéquat empêchent le fœtus d'atteindre son potentiel de croissance intrinsèque. Les enfants nés avec un RCIU sont ainsi plus susceptibles de développer à l'âge adulte des désordres métaboliques (revue Berends *et* Ozanne, 2012). En France, le RCIU représente 8% des naissances. Les données expérimentales obtenues sur ces modèles rongeurs de restriction/surcharge nutritionnelle ou calorique pendant la période de gestation et/ou de lactation ont permis de confirmer l'hypothèse de Barker, d'objectiver le concept de DOHaD (revue, McMullen *et* Mostyn, 2009) et continuent à apporter des connaissances sur les mécanismes sous-jacents (revue, Ramirez *et al.*, 2023).

Avant d'être recrutée pour développer un projet sur le rôle de l'axe intestin-cerveau dans les défauts de comportement alimentaire observés à l'âge adulte dans le modèle RCIU, j'ai

travaillé avec J.P. Segain, chercheur dans l'unité, sur l'impact du RCIU sur la fonction barrière intestinale. Son altération était supposée jouer un rôle dans les dérégulations métaboliques (Cani *et* Delzenne, 2009) ce qui a été largement confirmé depuis avec l'essor des analyses du microbiote intestinal (Revue, Régnier *et al.*, 2021).

A. LA PROGRAMMATION MÉTABOLIQUE DU CÔLON

Collaboration avec JP Segain dans le cadre de mon CRCT et qui s'est poursuivi au-delà de mon recrutement.

1. LE TISSU ADIPEUX VISCÉRAL ALTÈRE L'INTÉGRITÉ DE L'ÉPITHÉLIUM COLIQUE DANS UN MODÈLE DE RCIU

Lorsque l'UMR PhAN m'a accueillie en CRCT, Jean-Pierre Segain m'a proposé de travailler sur un projet original sur lequel il avait des données préliminaires et qui visait à explorer les effets du tissu adipeux viscéral sur l'intégrité de l'épithélium colique en réalisant des greffes de tissu adipeux sur la paroi colique *in vivo*. Il m'a également donné l'opportunité de co-encadrer avec lui le doctorant qui débutait alors sa thèse sur la programmation colique (Vianney Haure-Mirande, **thèse soutenue le 7/06/2013)**. Cette étude a généré des résultats originaux, publiés dans *Faseb Journal* en 2014 (Publication# 9, Annexe1, Le Dréan *et al.*, 2014**, V. Haure-Mirande, doctorant associé**) montrant que le tissu adipeux et la leptine augmentaient la perméabilité intestinale par un mécanisme dépendant des Rho-kinases. La publication de ce travail a donné lieu à la rédaction d'un commentaire sur invitation dans Tissue Barriers. Dans ce commentaire nous avons tenté, avec Jean-Pierre Segain, de mettre en lumière le rôle de la leptine à la fois dans le métabolisme énergétique et la régulation de la barrière épithéliale via la signalisation AMPK (AMP-activated protein kinase) (Publication #8, Le Dréan *et* Segain, 2014).

2. PROGRAMMATION FŒTALE DES PATHOLOGIES COLIQUES

Ma collaboration avec JP Segain s'est poursuivie mais de façon moins soutenue dès lors que j'ai obtenu un financement pour mon propre projet. Je suis néanmoins associée à un travail (publication#6, Desir-Vigne *et al.*, 2018, **V. Haure-Mirande associé**) dont l'objectif visait à corriger les effets négatifs du RCIU sur la muqueuse colique par un traitement de la mère gestante et allaitante en phénylbutyrate/glutamine (PG) (**Figure 1**).

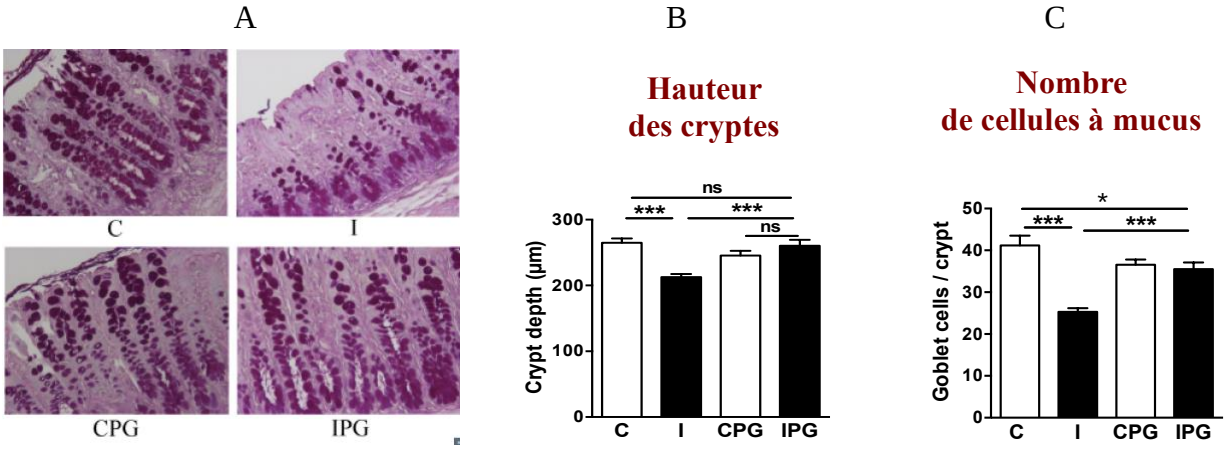


Figure 3. Effet d'un traitement au phénylbutyrate/glutamine (PG) des mères sur la muqueuse colique de la descendance mâle. A) Parois coliques de ratons nés de mères contrôle (20% de protéines, C), restreintes (8% de protéines, I) supplémentées en PG (CPG et IPG)

colorées à l'acide periodique Schiff (cellules à mucus) ; Histogramme montrant l'effet sur B) la hauteur des cryptes et C) le nombre cellules à mucus (n=8, *, *P<0,05*, ***, *P<0,001*).

Ma contribution à ce travail se positionnait en amont, dans le cadre de la thèse de Vianney Haure-Mirande, en ayant fourni des données (puces à ADN Illumina) montrant l'expression de marqueurs du stress du réticulum endoplasmique (forme épissée de XBP1, IRE1, Oasis…) dans le côlon de rats RCIU et une expression exacerbée de ces marqueurs sous une diète hyperlipidique (résultats non publiés).

B. IMPACT DE LA NUTRITION PÉRINATALE ET DU MICROBIOTE PRÉCOCE SUR L'AXE INTESTIN-CERVEAU, LA FONCTION ENDOCRINE INTESTINALE ET LE COMPORTEMENT ALIMENTAIRE ADULTE

Travaux menés à partir de mon recrutement à l'UMR PhAN en 2010

1. INTRODUCTION GÉNÉRALE

L'étude des effets de la nutrition périnatale sur l'axe intestin-cerveau représentait au moment de mon recrutement une réelle opportunité de valoriser l'ensemble des compétences acquises au cours de ma formation doctorale, postdoctorale et durant ma carrière d'enseignant-chercheur. En effet, au cours de ma thèse, j'avais travaillé sur la régulation nutritionnelle de la sécrétion pancréatique du jeune bovin par la cholécystokinine (CCK), peptide majeur de l'axe intestin cerveau. Mon séjour à l'UMR CNRS/Université de Rennes1 avait ensuite consolidé mes compétences en neuroendocrinologie et m'avait déjà ouvert les « portes » du système nerveux central et notamment l'hypothalamus.
Au cours des 13 dernières années, j'ai ainsi étudié la programmation de l'axe intestin-cerveau par des manipulations nutritionnelles de la mère ou par des modulations du microbiote néonatal des nouveau-nés avec comme « read out » le comportement alimentaire. Lorsque j'ai débuté ce projet à PhAN, ma vision de l'axe intestin-cerveau était focalisée sur la communication entre ces deux organes à partir des cellules entéro-endocrines (CEE) qui synthisent des peptides également produits par le cerveau comme par exemple la sérotonine ou la CCK, et qui utilisent les afférences vagales pour relayer les signaux périphériques vers le tronc cérébral. Mes hypothèses étaient donc fondées sur cette vision. Or l'axe intestin-cerveau inclut aussi le microbiote intestinal considéré à présent comme un organe à part entière (Morand, 2017). Aussi, les questions relatives aux effets « programmateurs » de la nutrition périnatale sur le comportement alimentaire adulte ne peuvent pas se limiter aux seuls effets sur l'intestin et le cerveau au moment de leur développement ou sur la voie vagale de communication entre eux. Ma collaboration avec ma collègue Catherine Michel a permis d'ajouter à mes hypothèses celle d'une programmation de la composition du microbiote intestinal en début de vie et qui perdure à l'âge adulte ou encore celle du microbiote comme vecteur d'une programmation (Michel *et* Blottière, 2022). Aussi, mes travaux sur la programmation des CEE chez le rat ont été abordés principalement par (i) les effets d'une restriction protéique maternelle pendant la gestation et la lactation, modèle sur lequel des défauts de régulation de la prise alimentaire avaient été préalablement rapportés au laboratoire (Orozco-Solis et al., 2009; Coupé et al., 2010), (ii) les effets d'une modulation néonatale du microbiote soit en apportant aux ratons des oligosides prébiotiques pendant la lactation, soit en implantant chez des ratons nouveau-nés des microbiotes de compositions différentes. Les effets sur le comportement alimentaire plus tard dans la vie, en lien avec la composition du microbiote en début de vie et à l'âge adulte, ont été étudiés en plus des effets sur la fonction endocrine intestinale.

Ces travaux ont été portés par 3 financements : PARIMAD, Olygose et Mamiprooffi. Le premier, PARIMAD (financement Région des Pays de La Loire, 2014-2017) abordait les effets de la dénutrition périnatale sur l'axe intestin-cerveau à travers les principaux acteurs régulant la prise alimentaire et les fonctions digestives : les CEE impliquées dans le «sensing nutritionnel» intestinal et la sécrétion de peptides régulateurs de l'appétit ; le système nerveux entérique (SNE) qui régule les fonctions digestives; la voie vagale qui intègre les signaux neuroendocrines vers le tronc cérébral et les centres hypothalamiques homéostatiques. Ce projet intitulé « Impact de la dénutrition périnatale sur l'intestin neuroendocrine » impliquait les 2 principales unités de recherche de l'Institut des Maladies de l'Appareil Digestif (IMAD), l'UMR PhAN et l'U1235 TENS INSERM (Directeur : M. Neunlist) dont la thématique porte sur le système nerveux entérique et les dysfonctions de barrière. Le département Alimentation Humaine d'INRAE avait soutenu ce projet en finançant à 50% la thèse de **M. Ndjim que j'ai co-encadrée avec M. Neunlist. J'ai également encadré une étudiante en master2, Camille Poinsignon sur ce projet.**

Deux autres projets menés entre 2017 et 2021 ont ensuite étudié la composante microbiote de l'axe intestin-cerveau en collaboration avec C. Michel. La question commune à ces deux projets, un financé par un industriel (contrat de collaboration Olygose 2015-2016) et le second par une ANR (Mamiprooffi, 2018-2021 portée par P. Parnet), visait à étudier les effets à court et à long terme d'une modification de la composition du microbiote en début de vie sur le comportement alimentaire adulte, en adressant plus particulièrement pour ma part la fonction endocrine intestinale comme piste mécanistique. **J'ai co-encadré A.L. Pocheron qui a réalisé sa thèse dans le cadre de ces deux projets ainsi que 4 étudiant.e.s en Master2 (Astrid Olier, Edith Gouyon, Bastien Houssais, Kourosh MoussaviI Seresht)**.

Plus récemment, je me suis fortement impliquée dans le projet GDM-Milk porté par ma collègue M.C. Alexandre-Gouabeau. Ce projet vise à étudier le cas très particulier de programmation métabolique qu'est le diabète gestationnel et à considérer la lactation comme un levier d'action permettant de limiter le risque de diabète de type 2 chez la descendance. J'interroge dans ce projet la question des effets des composés bioactifs du lait maternel sur la production d'incrétines par les CEE dans un modèle murin de DG et par approches *in vitro* sur des lignées de CEE humaines. Nous avons obtenu pour ce projet un financement de l'ANR (# ANR 22 CE17-0039). **Je co-encadre avec MC Alexandre-Gouabeau, P. Bobin qui réalise sa thèse sur ce projet, ainsi que 2 étudiant.e.s en master2 (Marie Michelland et Corentin Massias).**

2. L'AXE INTESTIN-CERVEAU DANS LA RÉGULATION HOMÉOSTATIQUE DE LA PRISE ALIMENTAIRE

a) Effet d'une restriction protéique maternelle sur le relais vagal, voie de communication majeure de l'axe intestin-cerveau, et sa régulation par la cholécystokinine, l'hormone de la satiété

(1) Positionnement du projet et hypothèses

La prise alimentaire est finement régulée à la fois par des mécanismes (i) homéostatiques, basés sur des réponses réflexes suite à l'arrivée des nutriments dans le tube digestif et qui impliquent l'intestin, le nerf vague et le tronc cérébral, et (ii) hédoniques qui font appel à la mémoire et l'apprentissage, fonctions supérieures gérées par le système nerveux central. Ces deux voies modulent le centre régulateur de la prise alimentaire, l'hypothalamus. La régulation homéostatique de la prise alimentaire abouti à l'état de non-faim, la satiété. La mise en place de la satiété (le rassasiement) repose sur l'action centrale d'hormones sécrétées par les CEE en

réponse à la distension gastrique et à l'arrivée d'aliments dans le tube digestif (revue Badman *et* Flier, 2005). Initialement décrits pour leur rôle dans la digestion et l'absorption des nutriments, ces peptides agissent sur les noyaux de l'hypothalamus impliqués dans la régulation de la prise alimentaire par la voie hormonale et aussi par l'intermédiaire des afférences vagales qui remontent les signaux sensoriels vers le tronc cérébral.

La CCK est considérée comme le peptide gastro-intestinal de la satiété à court-terme. Synthétisée et sécrétée par les cellules I du duodénum, cette hormone est connue depuis longtemps pour stimuler la sécrétion pancréatique exocrine dans le duodénum, la contraction de la vésicule biliaire et contrôler la vitesse de vidange gastrique. La CCK active les fibres vagales qui innervent l'estomac et le duodénum et son rôle sur la satiété se manifeste par une réduction de la taille du repas et de sa durée mais est sans effet sur l'intervalle entre 2 repas ou la taille du repas suivant (revue Moran, 2006). Les effets de la CCK sur la régulation de la prise alimentaire passent essentiellement par la voie vagale ; cela a largement été démontré chez plusieurs espèces dans la littérature (revue Raybould, 2007). Le nerf vague joue un rôle majeur dans l'axe intestin-cerveau puisqu'il transmet l'information sensorielle (distension gastrique, sécrétion de peptides gastro-intestinaux, etc.) vers les centres régulateurs de l'homéostasie énergétique (tronc cérébral et hypothalamus) (**Figure 4**). Les neurones afférents du vague expriment une grande diversité de récepteurs aux peptides gastrointestinaux (récepteurs CCK-1, récepteurs à la leptine, à la ghréline, au PYY…) et de neuropeptides (NPY, CART, MCH, CCK, ...).

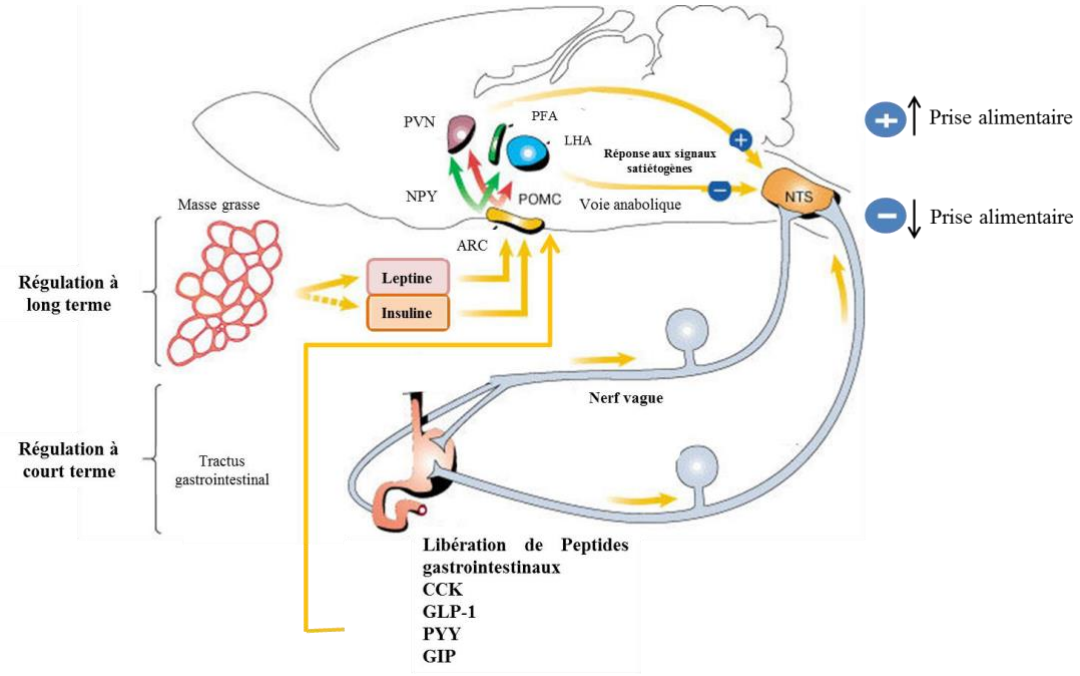


Figure 4. Régulation de la balance énergétique et de la prise alimentaire par les signaux périphériques en provenance de la sphère digestive.

Au moment où ce projet démarrait, un ensemble de données démontrait l'existence d'un switch de ce « phénotype neurochimique » du nerf vague régulé par la CCK (revues Dockray, 2009, Dockray, 2012). Il était proposé qu'à faible concentration plasmatique de CCK (restriction énergétique, phase de jeûne), le phénotype vagal contribuait à la stimulation de l'appétit

[expression des récepteurs MCH-1 et de MCH (inhibition des récepteurs au PYY et de CART)] alors qu'après un repas, la concentration plasmatique de CCK augmentant, celle-ci induisait un phénotype conduisant à la satiété [expression des récepteurs au PYY et de CART (inhibition de MCH-1 et de MCH)]. Ainsi le phénotype des neurones afférents du nerf vague pouvait passer d'un phénotype orexigène vers un phénotype anorexigène selon les besoins énergétiques de l'organisme (**Figure 5**).

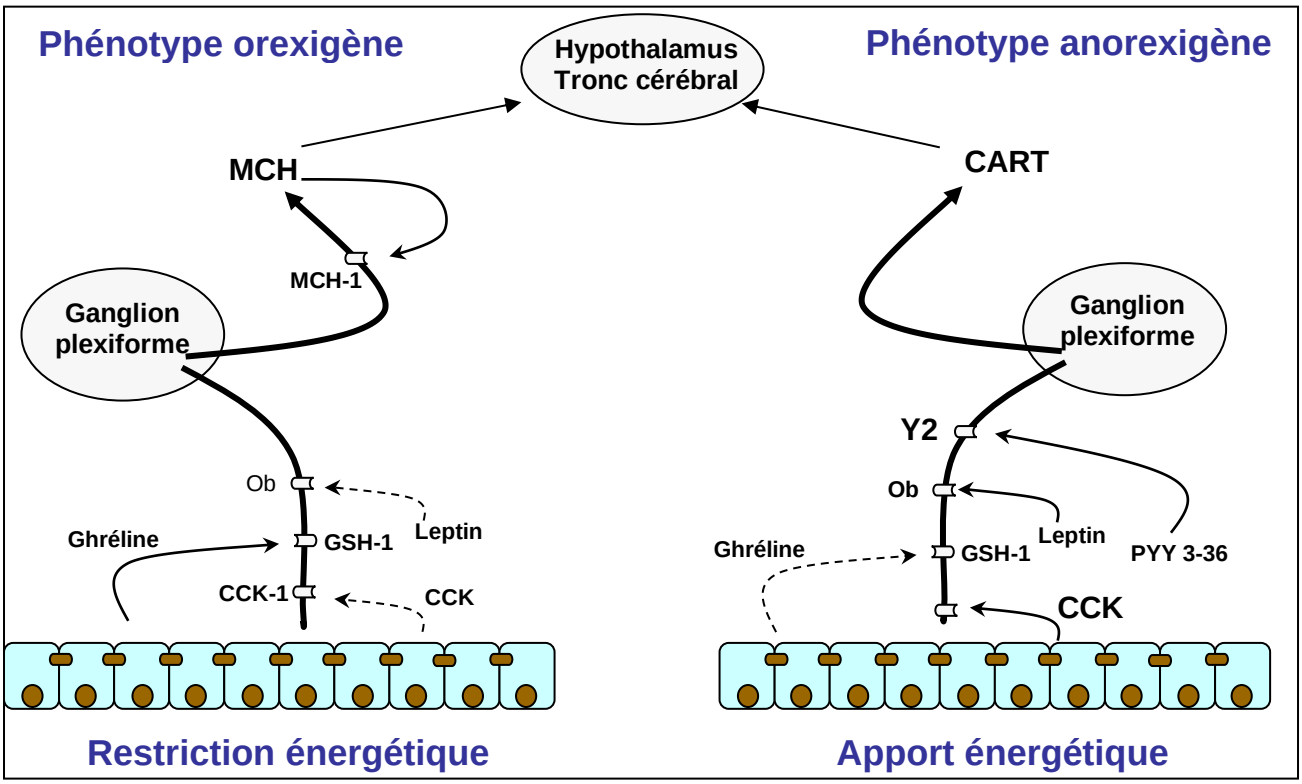


Figure 5. Axe intestin-cerveau : représentation schématique du switch neurochimique vagal contrôlé par la CCK.
GSH-1 : récepteur à la ghréline (GH : hormone de croissance) de type 1, CCK-1 : récepteur à la CCK de type 1, PYY : peptide YY, Y2 : récepteur au PYY, Ob : récepteur à la leptine, CART : cocaine-and -amphetamine regulated trancript, MCH : melanin concentrating hormone, MCH-1 : récepteur à MCH de type 1.

De façon intéressante, d'autres études montraient que cette sensibilité vagale à la CCK diminuait dans un modèle d'obésité induite par un régime hyper-énergétique (DIO, diet-induced obesity) (de Lartigue *et al.*, 2011 ; Duca *et al.*, 2013) et contribuait à l'hyperphagie observée dans ces modèles. Dans le modèle de rat né avec un RCIU par restriction protéique maternelle, il avait été observé une désorganisation du système neuropeptidergique hypothalamique, conduisant à une résistance aux effets satiétogènes de la leptine et de l'insuline persistante à l'âge adulte (Plagemann, 2006 ; Coupé *et al.*, 2010). Après le sevrage, la transition nutritionnelle s'accompagnait d'une modification du comportement alimentaire (hyperphagie) qui induisait une augmentation de la taille et de la durée du repas, et ainsi une satiété retardée (Orozco-Solis *et al.*, 2009).
J'avais alors proposé l'hypothèse que chez les rats nés de mères restreintes en protéines, cette sensibilité vagale à la CCK pouvait être altérée, affectant ainsi le processus de régulation à court-terme de la prise alimentaire à l'âge adulte.

(2) Stratégie et résultats principaux

Pour répondre à cette hypothèse, nous avons utilisé le modèle de restriction protéique maternelle (8% de protéines pendant la gestation et la lactation) chez le rat mâle adulte (160j). Le groupe témoin était constitué de rats nés de mères normo-protéiques (20% de protéines) (**Figure 6**).

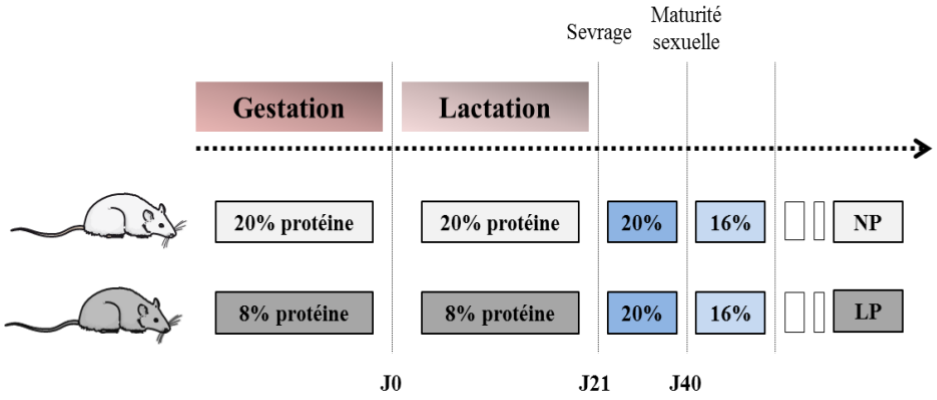
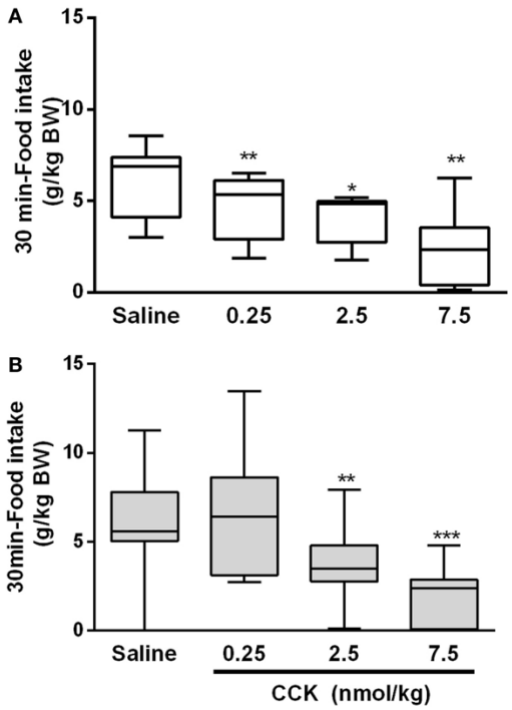


Figure 6. Protocole expérimental du modèle de rats dénutris en protéines en période pré- et postnatale.
NP (normo protein) correspond au groupe contrôle et LP (low protein) correspond au groupe de rats dénutris (RCIU). J0 = naissance, J21 = âge au sevrage et J40= âge à la maturité sexuelle

Une étude de comportement a d'abord été menée en cage physiologique afin d'obtenir des indications précises et continue sur 24h de la consommation des animaux mais également sur la séquence prandiale du premier repas effectué par les animaux après un jeune pour étudier le rassasiement et la satiété. Ensuite, une mesure de la cinétique de sécrétion de la CCK (à jeun/renourris) a été réalisée et la sensibilité aux effets satiétogènes de la CCK a été mesurée à l'aide d'injection à doses croissantes de CCK-8S (**Figure 7**). L'activation vagale (phosphorylation ERK et CaMKII) a été mesurée par Western-Blot dans les ganglions plexiformes qui regroupent les corps des neurones vagaux afférents. Le phénotype vagal a été déterminé par RTqPCR et immunofluorescence sur la base des marqueurs publiés.



Nos résultats (publication # 7, Annexe 2, Ndjim *et al.*, 2017, **M. Ndjim doctorante et C. Poinsignon M2 associées**) ont montré une résistance aux effets satiétogènes de la CCK en lien avec une expression du récepteur CCK-1 diminuée dans le vague afférent et des niveaux de phosphorylation de CaMKII en conditions basales fortement réduits.

Figure 7. Mesure de la prise alimentaire en réponse à différentes doses de CCK chez le rat né avec un RCIU. **P<0,05 ; **P<0,01 ; ***P<0,001 ;* différences significatives avec le groupe saline (NaCl 0,9%). A, Goupe control (normo-protéique) ; B, groupe LP, low-protein (8%). Test de Friedman et des rangs signés de Wilcoxon (mesures répétées).

Cette perte de sensibilité vagale aux effets satiétogènes de la CCK pourrait contribuer aux dérégulations de prise alimentaires observées chez les rats nés avec un RCIU. En revanche, cette étude n'a pas permis de mettre en évidence une altération du « switch phénotypique » vagal vers un phénotype orexigène, telle que décrite dans des modèles d'obésité par d'autres équipes (revue Dockray, 2012) et qui constituait un volet de notre hypothèse de départ.

b) Effet de restriction protéique maternelle sur la fonction endocrine intestinale

(1) Positionnement du projet et hypothèses

Les cellules entéro-endocrines, sentinelles de la muqueuse intestinale

L'épithélium intestinal constitue une interface majeure avec l'environnement extérieur. Son auto-renouvellement rapide, en quelques jours, lui permettrait de s'adapter sans dommage cellulaire durable au contenu luminal (nutriments, microbiote, agents pathogènes/toxiques, etc.). Il est principalement constitué de 6 types cellulaires (**Figure 8**), tous issus de la division de cellules souches Lgr5+ résidentes de la crypte et ensemble, assurent l'absorption (entérocytes) et l'intégrité de la muqueuse (cellules à mucus, tuft et de Paneth).

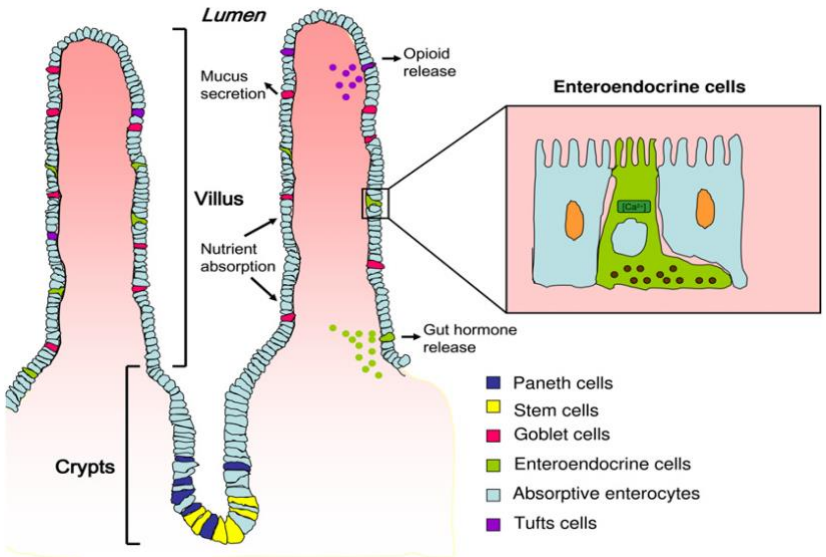


Figure 8. Schéma représentatif des principaux types cellulaires de la muqueuse intestinale. *D'après Moran-Ramos et al., 2012*.

Les CEE ne représentent que 1% de la population totale des cellules épithéliales mais constituent néanmoins l'organe endocrine le plus vaste de l'organisme. Elles sont équipées de récepteurs et de senseurs leur permettant de détecter un large répertoire de molécules de la lumière intestinale ou circulantes (nutriments, métabolites bactériens, bactéries, molécules irritantes, transduction du signal olfactif et du goût) et ainsi de transmettre les signaux périphériques aux systèmes immunitaires et nerveux entériques (Revues, Moran-Ramos *et al.*, 2012 ; Gribble *et* Reimann, 2016). Les CEE jouent ainsi un rôle clef dans la régulation de la réponse postprandiale en modulant la satiété et régulant les apports énergétiques en adéquation

avec les besoins (Revue, Sam *et al.*, 2012). Les peptides gastro-intestinaux qu'elles produisent ont des effets pléiotropes : motricité intestinale, absorption, sécrétions et métabolisme. Les CEE avaient été initialement classées selon le peptide majoritaire qu'elles produisent : le glucagon-like peptide 1 (GLP-1) et le peptide YY (PYY) sont sécrétés par les cellules L ; le glucose-dependent insulinotrophic polypeptide (GIP) par les cellules K ; la CCK par les cellules I ; la sérotonine par les cellules entéro-chromaffines, etc. Des données récentes basées sur l'utilisation de rapporteurs fluorescents chez la souris couplée à des approches transcriptomiques en Single Cell ont montré que cette nomenclature n'était pas le reflet du répertoire hormonal d'une CEE, capable en réalité de produire et sécréter plusieurs hormones. En effet, la différenciation des CEE peut prendre différentes trajectoires selon l'expression ou l'inhibition de l'expression de certains facteurs de transcription et selon leur position dans l'axe crypto-villositaire qui les expose à un gradient Wnt/ (bone morphogenic protein) BMP (Gehart *et al.*, 2019). Le destin des cellules issues du compartiment « souche » sera sécrétoire grâce à l'inhibition de Notch qui permet l'expression de Math1 (**Figure 9**). Ces précurseurs se destinent au lignage endocrine par l'expression de Ngn3 (Jenny *et al.*, 2002). Chez la souris, la spécialisation vers une production de CCK est dépendante de l'expression de NeuroD1, Nkx2.2, Pax4, Arx alors que la production de GLP-1 sera sous le contrôle de Pax6, Foxa1 et Foxa2 (Revues, Schonhoff *et al.*, 2004 ; Li *et al.*, 2011).

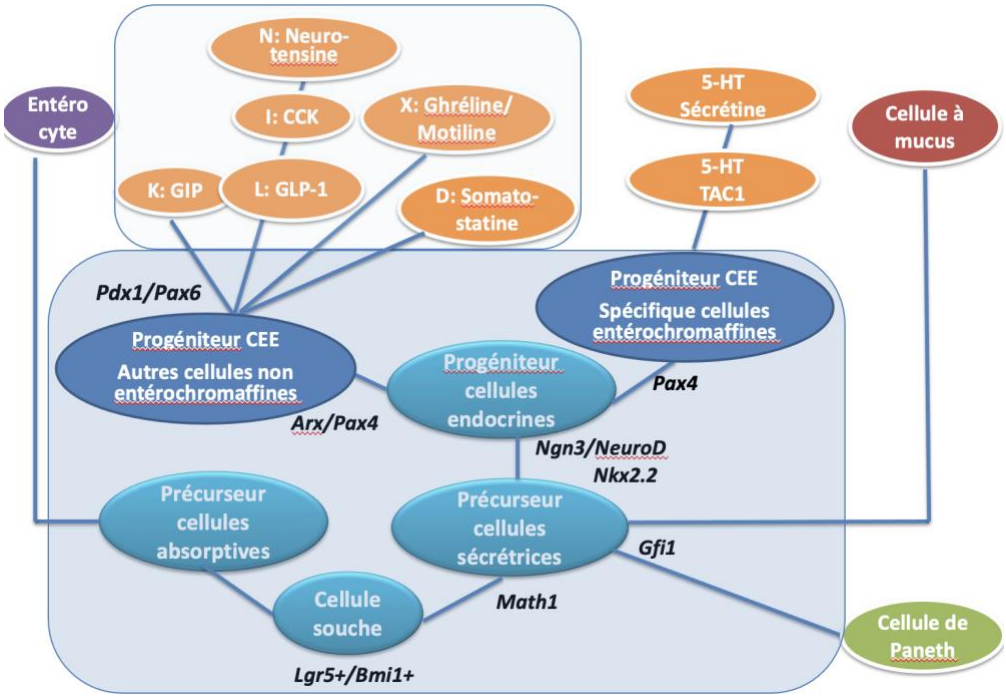
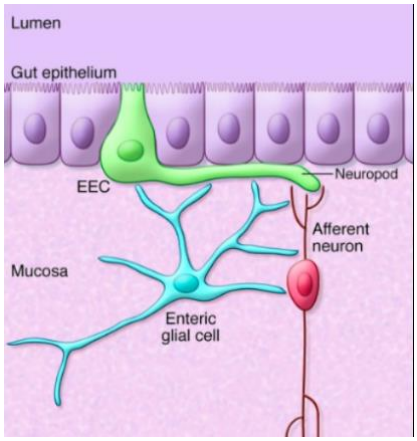


Figure 9. **Destin cellulaire de la cellule souche intestinale vers le lignage sécrétoire ou absorptif (entérocytes) et sa régulation par les facteurs de transcription au cours de la différenciation des cellules épithéliales intestinales.** Les 5 lignages entéro-endocrines actuellement retenus (orange) proviennent d'un progéniteur endocrine commun exprimant neurogenin 3 (Ngn3). 5-HT : sérotonine, Tac-1 : Tachykinin precursor 1.

Dans la muqueuse intestinale, les CEE de type ouvert sont polarisées. Leur pôle apical, en contact avec la lumière du tube digestif détecte la présence de nutriments et de métabolites bactériens grâce à l'expression d'un vaste répertoire de récepteurs/transporteurs/senseurs membranaires. Ce « sensing » déclenche des voies de signalisation bien connues (protéines G, alpha-gustducin…) menant à la sécrétion des hormones au pôle basolatéral qui vont agir sur de nombreuses fonctions digestives et métaboliques. Les CEE sont également en étroit contact



avec le système nerveux entérique (SNE) et les afférences vagales grâce à leurs prolongements cytoplasmiques (neuropodes, **Figure 10**) à partir desquels une communication de type synaptique a été proposée (Borhorquez *et* Liddle, 2015) puis mise en évidence (Bellono *et al.*, 2017, Kaelberer *et al.*, 2018). Ces cellules constituent donc des acteurs précoces et majeurs de l'axe intestin-cerveau.

Figure 10. **Schéma représentant la connexion entre CEE et SNE, appelé aussi « gut connectome »** (extrait de Borhorquez et Liddle, 2015)

Au moment où démarrait ce projet, l'impact de la nutrition périnatale sur une potentielle « programmation » des CEE n'était que peu documenté. Une étude chez le porc né avec un RCIU montrait une diminution de CEE duodénales à la naissance mais qui ne perdurait pas au-delà de 14j, selon le type d'allaitement (Radlowski *et al.*, 2014). Une restriction calorique post-natale augmentait le nombre de cellules entéro-chromaffines (sérotonine) dans le côlon chez le rat (Schoffen *et al.*, 2014). Par ailleurs, dans un contexte de stress de séparation maternelle, le nombre de CEE était augmenté chez le rat adulte (Estienne *et al.*, 2010). En revanche, de plus nombreuses études avaient montré que d'autres cellules épithéliales intestinales de la lignée sécrétoire (cellules à mucus, cellules de Paneth) étaient sensibles à la dénutrition périnatale rapportant une diminution des cellules à mucus iléales chez le porcelet RCIU nouveau-né (Wang *et al.*, 2005 ; Dong *et al.*, 2014) ou encore du nombre de cellules à mucus et des cellules de Paneth dans un modèle de souris née avec un RCIU (hypertension maternelle) (Fung *et al.*, 2016). Une restriction protéique maternelle pendant la seule période de gestation chez le rat diminuait le nombre de cellules à mucus dans le côlon distal mais l'effet ne perdurait pas au-delà de 40j (Fança-Berthon *et al.*, 2009). Plus tard, nous montrerons qu'une restriction protéique maternelle pendant la gestation et la lactation diminue significativement le nombre de cellules à mucus dans le côlon à l'âge adulte chez le rat (**Figure 3**, publication#6, Désir-Vigné *et al.*, 2018, **V. Haure Mirande associé**). Dans le jéjunum, cette diminution n'était observée que lorsque les rats nés avec un RCIU étaient soumis à une diète hyper-lipidique (publication#11, Lallès *et al.*, 2012). Les CEE étant issues du même précurseur de lignée sécrétoire que les cellules de Paneth et les cellules à mucus, il était concevable qu'elles soient elles aussi soumises de façon durable aux effets de la dénutrition périnatale. De plus, nous avions montré une sécrétion post-prandiale plus importante de CCK, produite par les CEE de type I chez les rats nés avec un RCIU adulte (**Figure 11**, publication#7, Annexe 2, Ndjim *et al.*, 2017, **C. Poinsignon M2 associée**), suggérant soit un nombre de CEE-I plus important chez les rats nés avec un RCIU (LP), soit une plus grande sensibilité de ces cellules à sécréter la CCK.

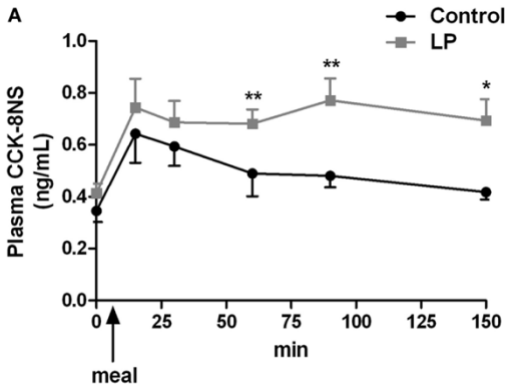


Figure 11. **Cinétique de sécrétion postprandiale de CCK (ELISA) chez des rats mâles adultes**. Control, régime maternel à 20% de protéines ; LP, low-protein 8% de protéines). (n=9). *, *P< 0,05* entre LP et Control

J'avais alors émis l'hypothèse que les défauts de régulation de prise alimentaire des rats nés avec un RCIU pouvaient être dus à un effet de la restriction protéique maternelle sur (i) la densité des cellules productrices de CCK dans le duodénum (i.e. sur leur capacité à proliférer ou se différencier à l'âge adulte, (ii) leur fonction de « sensing nutritionnel » (i.e. leur capacité à détecter les nutriments de la lumière intestinale) réduisant dans les 2 cas, la signalisation périphérique permettant la mise en place de la satiété.

(2) Stratégie et principaux résultats

(a) Prolifération et différenciation des CEE-CCK

Afin de répondre à ces questions, j'avais généré en 2015, en collaboration avec la plateforme Trip (Transgenèse rat, S. Rémy et L. Tesson, UMR1064, CR2TI) de la SFR F. Bonamy de Nantes des rats transgéniques KI pCCK-eGFP exprimant un gène rapporteur fluorescent (eGFP) placé sous le contrôle du promoteur du gène de la CCK (stratégie TALEN). Ce modèle KI permet d'étudier les CEE-CCK (GFP positives) *in situ* sans marquage préalable et devait surtout me permettre d'isoler et d'étudier spécifiquement cette population de CEE natives, très faiblement représentée dans la muqueuse intestinale (**Figure 12**). Des rats CCK-GFP nés avec un RCIU ont été générés comme précédemment (**Figure 6**) pour constituer un groupe NP (normo-protein) et LP (low-protein).

A B

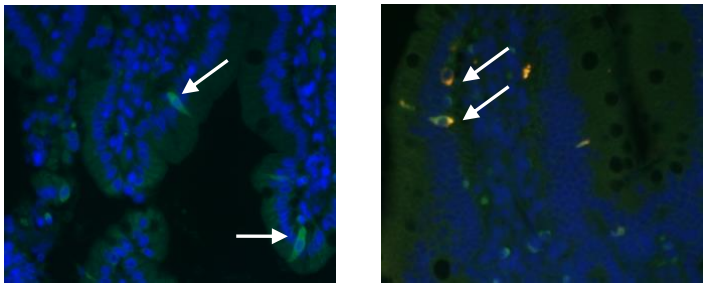


Figure 12. **Clichés illustrant (A) l'expression de la CCK (en vert) dans les CEE-I (flèches) des villosités du duodénum de rats pCCK-GFP.** Coloration des noyaux au Dapi (en bleu). La forme caractéristique « en amphore » des CEE ouvertes sur la lumière intestinale est bien visible sur ces coupes. En B, un immunomarquage dans le rouge à l'aide d'un anticorps dirigé contre la chromogranine A, exprimé par la plupart des CEE matures, montre que les CEE-I expriment également la chromogranine A (flèche). Grossissement x20.

Les résultats obtenus par **M. Ndjim** durant sa thèse montraient une plus forte densité de CEE totales (Chromogranine A positives) et des CCK-GFP positives dans les villosités duodénales de rats CCK-GFP nés avec un RCIU. La hauteur des villosités était également augmentée si bien que le ratio CEE-CCK/CEE totales n'était pas modifié (**Figure 13**).

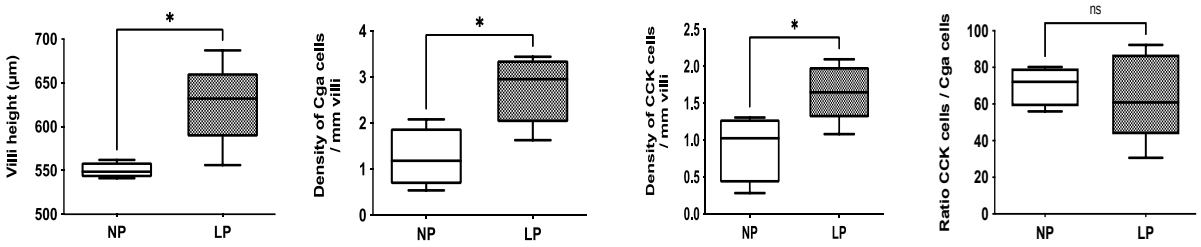


Figure 13. Impact du RCIU (groupe LP) dans le duodénum sur A) la hauteur des villosités, B) la densité des CEE totale (positives pour la chromogranine A (CgA), C) la densité des cellules positives pour la CCK et D) le ratio entre cellules CCK et CEE totales. *, *P<0,05*, différence significative avec le groupe témoin (NP).

Des analyses immunohistochimiques des activités de prolifération (PCNA) dans les cryptes ou d'apoptose (caspase 3) à l'apex des villosités sur la muqueuse duodénale n'ont pas montré de différence entre les groupes NP et LP. Des analyses transcriptomiques n'ont pas permis de mettre en évidence des différences d'expression des facteurs de transcription évoqués précédemment probablement en lien avec la très faible représentation des CEE-CCK (entre 0,3 et 0,5% des cellules épithéliales totales). L'objectif principal de cet outil (rat pCCK-GFP) était de trier les cellules GFP+ et de pouvoir ensuite mener une analyse transcriptomique en single cell sur une population purifiée de CEE-CCK. Malheureusement, nous ne sommes pas parvenues à lever le verrou technologique de l'extraction et la purification de ces cellules par tri au Facs.

En 2019, j'ai relancé une expérimentation animale en produisant des rats pCCK-GFP nés avec un RCIU afin de tenter la purification des pCCK-GFP par microdissection laser (collaboration plateforme d'imagerie ONIRIS, Dr T. Larcher). Là encore, mes tentatives se sont soldées par un échec vraisemblablement lié à une intensité de fluorescence trop faible pour être détectée sur tissu non fixé. L'évolution des protocoles permettant l'extraction d'ARN à partir de tissus fixés m'amène à reconsidérer cette technique aujourd'hui après marquage des CEE à l'aide d'un anticorps spécifique (Chromogranine A ou peptide d'intérêt) en collaboration avec la plateforme Micropicell (UMS Biocore, Nantes).

(b) Fonction « sensing nutritionnel » des CEE-CCK

Les cellules I sécrètent de la CCK en réponse à l'arrivée des nutriments dans le duodénum et notamment les acides gras à chaînes moyennes et longues (> 12 C). Notre premier objectif était de tester cette fonction de sensing lipidique des CEE-CCK dans notre modèle de rat RCIU en prenant en référence l'effet d'un gavage oral de ces acides gras sur la prise alimentaire. Une étude montrait en effet que le palmitoléate stimulait la sécrétion de CCK chez le rat de 2 mois et avait des effets inhibiteurs sur la prise alimentaire (Yang *et al.*, 2013). Nous attendions une altération de la réponse satiétogène chez les rats RCIU. Or, nous n'avons déjà pas reproduit l'effet inhibiteur de la prise alimentaire chez les rats contrôles (NP, normoprotein) malgré des conditions expérimentales similaires (dose, âge des animaux, etc.) et la concentration plasmatique de CCK était significativement diminuée alors qu'elle augmentait dans le papier de référence (Yang *et al.*, 2013). En revanche, notre étude a montré que le palmitoléate semblait promouvoir la satiété chez les rats RCIU (LP, low protein) en lien avec une augmentation de la concentration plasmatique en CCK pendant les 90 min post gavage (**Figure 14**).

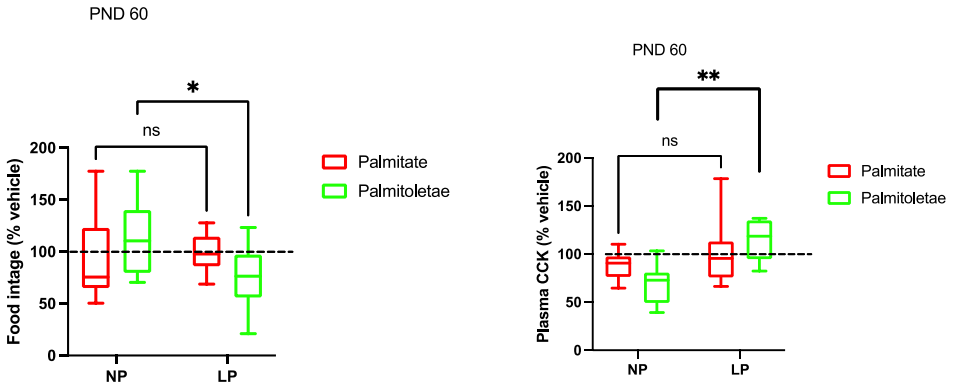


Figure 14. Effet d'un gavage en palmitoléate sur la prise alimentaire et la concentration plasmatique en CCK chez des rats nés avec un RCIU (LP, low protein) et contrôles (NP, normoprotein) à 60 jours. PND : postnatal day. *, *P<0,05*, **, *P<0,01*, analyse de variance à 2 voies et test de Sidak.

Le sensing des acides gras à chaîne longue (AGCL) par les CEE étant médié par des récepteurs spécifiques, FFAR1 et FFAR4 (Free-Fatty Acid receptor -1 et -4), nous avons recherché l'expression de ces récepteurs dans le duodénum. L'expression de ces deux senseurs était significativement augmentée dans le groupe LP traité au palmitoléate en comparaison avec le groupe NP (**Figure 15A**), ce qui pourrait signifier une plus sensibilité à cet AG dans le groupe LP. L'absence d'effet du palmitate pourrait être lié à une moins bonne absorption de cet AGCL dans le duodénum, comme en témoigne les teneurs en AGCL dans ce tissu après traitement (**Figure 15B**). En effet, les teneurs en palmitoléate étaient significativement augmentées dans le duodénum des rats traités au palmitoléate alors que le traitement au palmitate était sans effet sur sa concentration dans le duodénum.

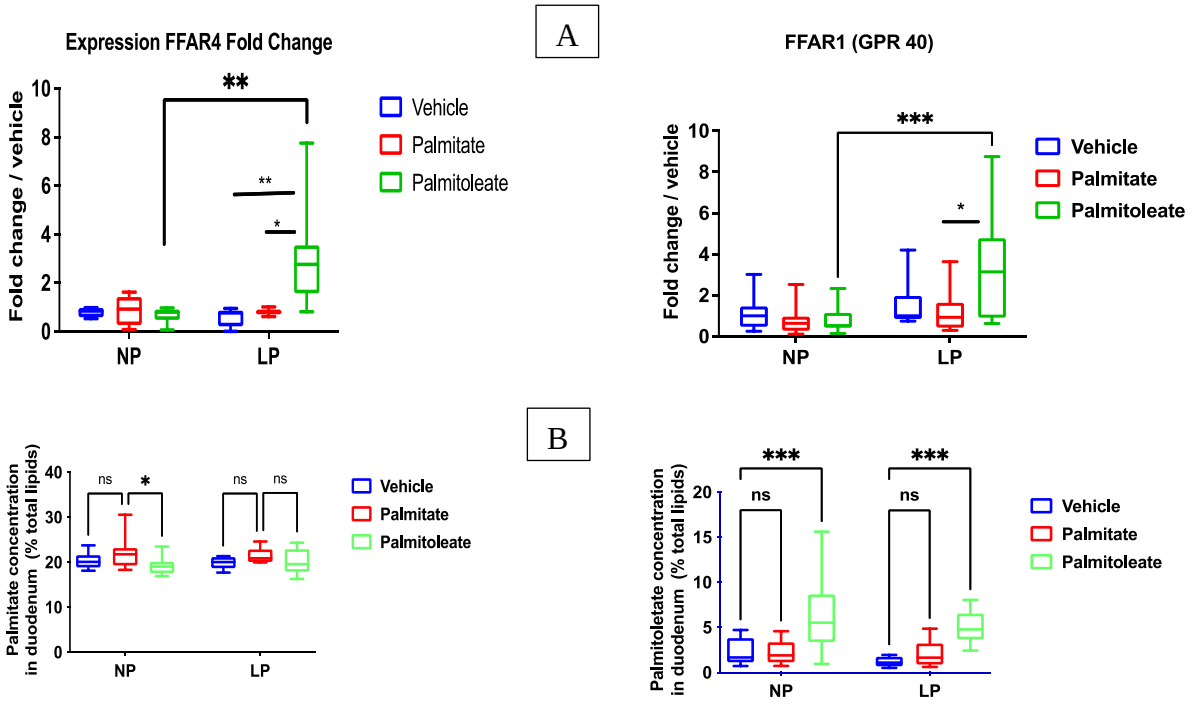


Figure 15. Effet d'un bolus de palmitate ou de palmitoléate sur A) l'expression des FFAR-1 et -4, B) leur absorption (concentration tissulaire) dans le duodénum de rat LP vs NP. *, *P< 0,05*, **, *P<0,01*, ***, *P<0,001*, différences significatives entre les groupes indiqués.

Ce travail sur la programmation de sensing nutritionnel des CEE-CCK a généré des résultats originaux mais inattendus. En effet, l'objectif était de montrer un défaut de sensing nutritionnel dans ces cellules qui pourrait expliquer le retard à la satiété observé dans nos modèles de rats nés avec un RCIU. Or nous avons plutôt observé l'inverse. Cependant, le fait de ne pas reproduire les effets satiétogènes du palmitoléate dans le groupe NP rendait difficile l'interprétation de ces résultats. Ces effets pourraient néanmoins être discutés par rapport à l'âge des animaux, le choix du nutriment ou encore le moment de l'analyse post-gavage. Le fait d'observer une meilleure réponse à la stimulation par le palmitoléate chez des rats nés avec un RCIU à 60 jours pourrait se concevoir à travers une adaptation des mécanismes de régulations de la prise alimentaire, très majoritairement orientés vers une inhibition, qui permettrait une réponse aux besoins des animaux en croissance. Il est en effet connu qu'à ce stade de développement, les rats sont moins sensibles aux effets satiétogènes des peptides gastro-intestinaux tels que la CCK (Balasko *et al.*, 2012). Cependant à un âge plus avancé (130 jours), la stimulation aux AGCL (palmitoléate) n'a eu aucun effet, ni sur la prise alimentaire, ni sur la sécrétion de CCK (résultats non montrés). Des expérimentations auraient pu être montées en utilisant d'autres stimuli nutritionnels de la libération de CCK tels que les peptones. Notre choix s'était porté sur deux AGCL en raison de leurs effets publiés sur la prise alimentaire et la sécrétion de CCK chez le rat (Yang *et al.*, 2013). Enfin, la prise alimentaire avait été mesurée toutes les 30 min post-gavage jusqu'à 90 min car les effets satiétogènes de la CCK sont rapides après ingestion. Notre fenêtre d'effet attendu était similaire à celle utilisée dans ce type de protocole (Blevins *et al.*, 2008; de Lartigue *et al.*, 2012) mais il n'est pas à exclure que les effets sur la sécrétion de CCK se soient produits avant les 90 min post-gavage.

Ce travail réalisé par **M. Ndjim** a été valorisé par des communications à des congrès internationaux (**PO#18, P#19, P#20**) et nationaux (**PO#22 et PO#26, P#25**).

En conclusion générale, l'ensemble de ce travail mené en grande partie dans la thèse de **M. Ndjim** a permis de mettre en évidence que les défauts de régulation de prise alimentaire observés chez les rats nés avec un RCIU pouvaient être liés à une perte de sensibilité vagale à la CCK, peptide satiétogène produit par les CEE. L'adaptation de la fonction endocrine intestinale s'est manifestée par une augmentation de la densité de ces cellules et une meilleure sensibilité (mesurée très indirectement par la prise alimentaire post-gavage) aux effets satiétogènes de palmitoléate. L'effet du RCIU sur la fonction endocrine intestinale ne permet donc pas d'expliquer le retard de satiété dans ce modèle.

c) Effet de la restriction protéique maternelle sur les fonctions digestives

Dans le cadre du projet PARIMAD et de la thèse de M. Ndjim, en collaboration avec M. Neunlist, nous avons testé l'hypothèse selon laquelle les interactions CEE-SNE pouvaient être altérées par la restriction protéique maternelle, prédisposant à terme à des pathologies intestinales. De façon indirecte, nous avons étudié ces interactions par une approche fonctionnelle, en mesurant des paramètres de fonctions digestives régulées par le SNE : transit, motricité et perméabilité de l'intestin. Notre étude s'est focalisée sur le duodénum afin de garder le rationnel du sensing nutritionnel et des effets des nutriments dès leur arrivée dans l'intestin grêle. Les fonctions de motricité (Electric Field Stimulation, EFS) et de transit (migration rouge carlin et excrété fécal) n'étaient que peu ou pas modifiés chez les rats nés avec un RCIU (LP) par rapport aux rats témoins (NP) et les effets des acides gras restaient très modérés (non montrés).
En revanche, nous avions observé que le gavage en palmitoléate semblait augmenter la perméabilité paracellulaire *in vivo* et *ex vivo* mesurée en chambre d'Ussing chez les rats LP

mais pas chez les NP (**Figure 16**). Néanmoins pour observer cette tendance, il nous fallait considérer les groupes NP et LP indépendamment ce qui est discutable sur le plan statistique.

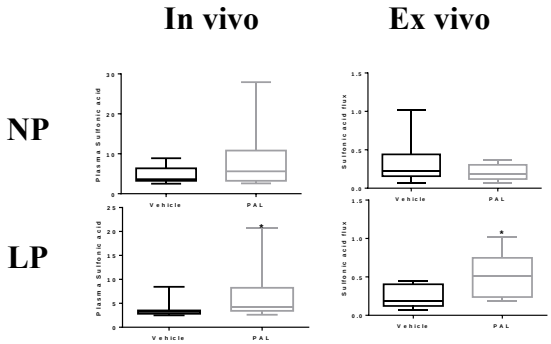


Figure 16. Perméabilité paracellulaire duodénale *in vivo* et *ex vivo* (chambre d'Ussing) en réponse à un bolus de palmitoléate chez les rats NP et LP à 60 jours. *, *P<0,05*, test de Mann-Withney.
NP, normo protein ; LP, Low protein

En effet, la très forte variabilité des données ne permettait pas d'envisager une ANOVA à deux voies. Les effets observés ne pouvaient donc être considérés que comme des tendances. Les mêmes observations avaient néanmoins été faites sur la perméabilité paracellulaire mesurée *in vivo* ainsi que pour la perméabilité transcellulaire et nous ont amenés à considérer le rôle de la CCK dans la fonction de perméabilité intestinale. En effet, sa concentration était augmentée après un bolus de palmitoléate chez les rats LP dans nos expériences mais également chez des rats NP dans une autre étude (Yang *et al.*, 2013). De plus, d'autres AGCL tels que l'oléate étaient connus pour stimuler la sécrétion de CCK (Liou *et al.*, 2011). Nous avons ainsi montré que sur des parois duodénales de rats standards adultes montées en chambre d'Ussing, la CCK induisait une augmentation de la perméabilité paracellulaire. Ces résultats originaux montraient pour la première fois un effet de ce peptide sur la fonction de perméabilité intestinale, qui serait dépendante de la voie cholinergique (**Figure 17**).

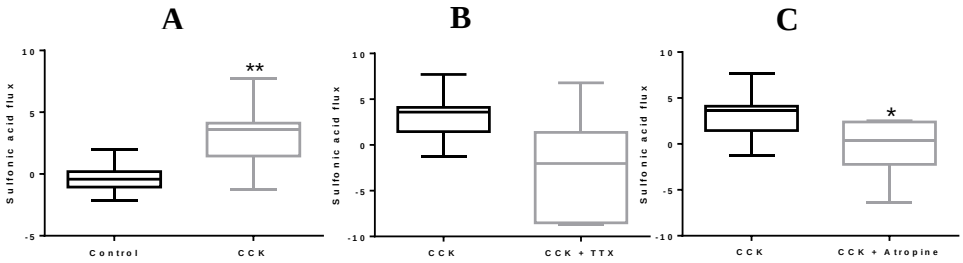


Figure 17. **Effet de la CCK sur la perméabilité paracellulaire mesurée en chambre d'Ussing sur des parois duodénales de rats âgés de 2 mois.** A : CCK (200 ng/mL), B : TTX, tétrodotoxine (1µM), C : atropine (1 µM) ajoutées dans le compartiment basolatéral 30 min avant l'acide sulfonique FITC (10mg/mL) ajouté dans le compartiment luminal. *, *P<0,05* ; **, *P<0,01*, test de Mann-Withney (n=11-16).

Ces résultats préliminaires ouvraient de nouvelles pistes de recherche sur les interactions entre les peptides gastrointestinaux produits par les CEE et la fonction de perméabilité régulée par le SNE. A la fin du projet PARIMAD, je n'ai pas pu donner de suite à ces observations faute de financements.

d) Une modulation néonatale de la composition du microbiote intestinal a-t-elle un impact sur le lignage entéro-endocrine et le comportement alimentaire adulte ?

(1) Modulation par supplémentation néonatale en oligosides prébiotiques

Dans le projet en collaboration avec Olygose, nous avons exploré les effets d'une supplémentation néonatale en prébiotiques sur le comportement alimentaire à l'âge adulte en focalisant sur les impacts à court terme (au moment des supplémentations) et à long-terme (à l'âge adulte) sur les CEE-L, sécrétrices de GLP-1 et de PYY. Ces 2 peptides gastrointestinaux produits par l'intestin distal (iléon-côlon) sont impliqués dans la régulation de la prise alimentaire et notamment la satiété. Des données chez l'homme adulte avaient montré qu'une supplémentation en prébiotique réduisait l'appétit via une augmentation de la production de GLP-1 (Cani *et al*., 2009).

Les oligosaccharides (OS) indigestibles étudiés étaient les suivants : alpha-galacto-oligosaccharides fournis par Olygose (αGOS), mélange de béta-galacto-oligosaccharides et d'inuline (GOS/In) ou fructo-oligosaccharides (FOS).

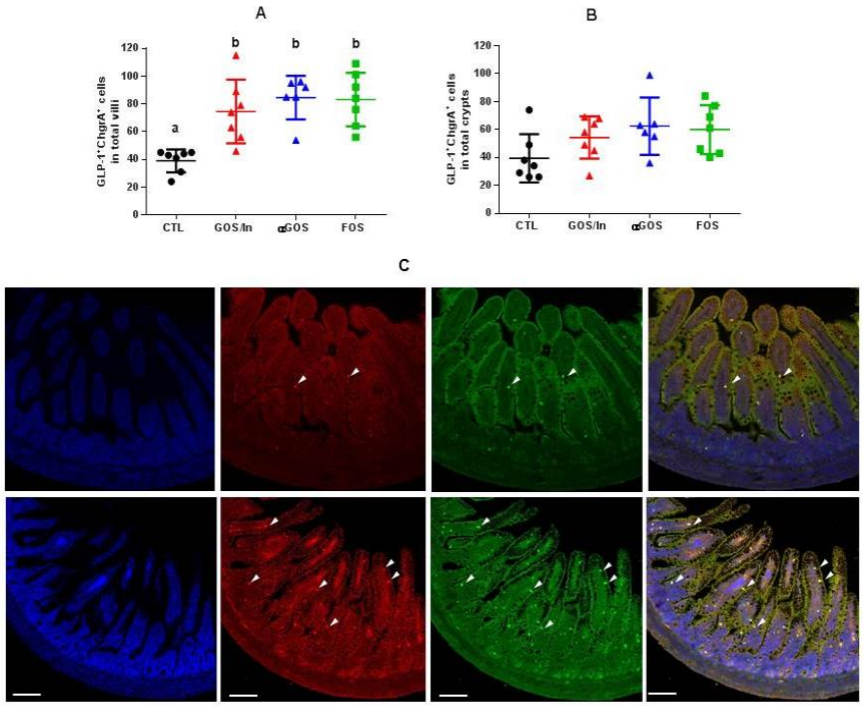


Figure 18. Une supplémentation néonatale en prébiotiques (OS) augmente la densité des CEE-L dans l'iléon. Dans les villosités, B) dans les cryptes ; a, b indiquent des différences significatives (*P< 0,05*) entre les groupes, moyenne ± SD (n=6-7 par groupe) C) Images représentatives de coupes d'iléons du groupe CTL (ligne supérieure) et αGOS (ligne inférieure), les flèches indiquent les cellules positives pour le GLP-1 (rouge) et la chromogranine A (vert). Echelle 100µm. (D'après publication #4, Annexe 3, Le Dréan *et al.*, 2019, **A.L Pocheron associée**).

Les principaux résultats de ce projet (Le Dréan *et al.*, 2019) ont montré que la supplémentation néonatale (entre 5 et 15 jours de vie) en OS prébiotiques a un effet immédiat sur la densité des cellules L (**Figure 18**) ainsi que sur l'expression des marqueurs de différenciation de la lignée entéro-endocrine, qui sont significativement diminués dans l'iléon (mais pas dans le côlon) des rats supplémentés (*vs* rats non supplémentés). Les taux plasmatiques de GLP-1 et de PYY étaient significativement augmentés en fin de supplémentation mais à l'âge adulte la sécrétion

plasmatique postprandiale de ces peptides n'était pas différente, que les rats aient été supplémentés ou non. De même, l'étude du comportement alimentaire (ingéré sur 24h, préférences pour le goût sucré, etc.) ne montrait pas d'effet majeur de la supplémentation néonatale en OS. Ces données semblent signifier que la modulation du microbiote intestinal en période néonatale par des supplémentations en OS n'a pas d'impact sur le comportement alimentaire à l'âge adulte. Ces résultats, rassurants en termes de sécurité des aliments, ne corroborent en revanche pas l'hypothèse selon laquelle une modulation néonatale du microbiote intestinal aurait des effets durables ou à distance sur le comportement alimentaire et la fonction entéro-endocrine à l'âge adulte. Ce travail a apporté de nouvelles connaissances opérationnelles concernant un nouveau prébiotique produit par la société Olygose et a fait l'objet d'un rapport. Les résultats ont été valorisés par des communications à des congrès, un pre-print dans NutriArchiv, un article publié en 2019 dans la revue Nutrients et dans les faits marquants du départements Alim-H sur cette période. **Une doctorante (Anne-Lise Pocheron) et deux étudiantes en M2 (Astrid Olier & Edith Gouyon) ont été co-encadrées par C. Michel et moi-même dans le cadre de cette étude.**

(2) Modulation par un transfert de microbiotes maternels de compositions différentes à des ratons nouveau-nés

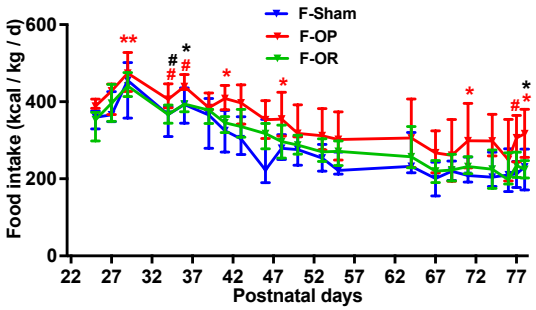
Dans le cadre de l'ANR Mamiprooffi (P. Parnet), nous avons interrogé le rôle du microbiote néonatal sur les troubles du comportement alimentaire au cours de la vie. Cette question se justifie par la concomitance de la colonisation massive du tube digestif du nouveau-né et la poursuite du développement du cerveau pendant la période postnatale (Ronan *et al.*, 2021). Ce projet visait donc à étudier l'impact de microbiotes maternels différents sur le comportement alimentaire de la descendance. Pour cela il nous fallait transférer à des ratons nouveau-nés (mères biologiques de souche Fischer) des microbiotes issus de mères donneuses pour lesquelles la composition du microbiote (intestinal) était connue pour être différente. Nous avons ainsi choisi les souches Sprague-Dawley Obese Prone (OP) et Obese Resistant (OR) qui répondent différemment à un régime hypercalorique, les premières restant minces et les secondes devenant obèses. Nos objectifs principaux étaient (1) de valider l'implantation de microbiotes différents chez les ratons receveurs et décrire son devenir au cours du temps (2) étudier le comportement alimentaire tout au long de la vie (3) rechercher des modifications structurelles et fonctionnelles des organes cibles, l'intestin et le cerveau (4) établir des liens de corrélations entre la composition du microbiote (au sevrage et à l'âge adulte) et les variables décrivant le comportement alimentaire, la fonction intestinale ainsi que les marqueurs de l'activité neurale dans les noyaux centraux impliqués dans la régulation du comportement alimentaire.

Initialement ma contribution à ce projet se situait en aval des objectifs présentés ci-dessus puisque je devais étudier la composante neuroendocrine intestinale en réponse à l'implantation de microbiotes de compositions différentes sur l'axe intestin-cerveau et le comportement alimentaire à l'âge adulte. Cependant, l'extrême lourdeur de l'expérimentation animale m'a amenée à travailler quasiment à temps plein sur ce projet financé pendant 4 ans et **à co-encadrer la thèse d'A-L Pocheron à 50%** (initialement prévu à 20%) avec P. Parnet.
J'ai également supervisé la mise au point de l'extraction d'ADN pour le séquençage 16S et shotgun à partir de matrices difficiles faiblement concentrés en microorganismes (lait, fluide vaginal) et de faibles volumes. **Deux étudiants de master2 ont participé à ce projet (Bastien Houssais et Kourosh Seyest)**.

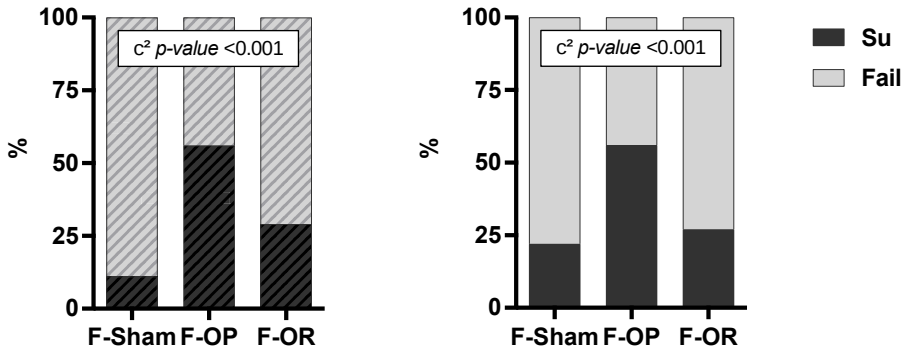
(a) Microbiotes maternels et Comportement alimentaire

Le résultat majeur de ce projet est que certains items du comportement alimentaire des receveurs différaient selon le microbiote reçu à la naissance et durant les 15 premiers jours de vie alors même qu'à l'âge adulte, il n'y avait plus de différences de composition du microbiote. Ainsi, les descendants femelles ayant reçu un microbiote de mère OP (F-OP) semblent exprimer des comportements de surconsommation sous régime chow (**Figure 19**A) ainsi qu'une plus forte motivation à consommer (**Figure 19B**), ce qui pourrait contribuer à un phénotype obésogène alors que les descendants des mères OR (F-OR) ont consommé moins de solutions aux goûts gras et sucré (**Figure 19C**)

A



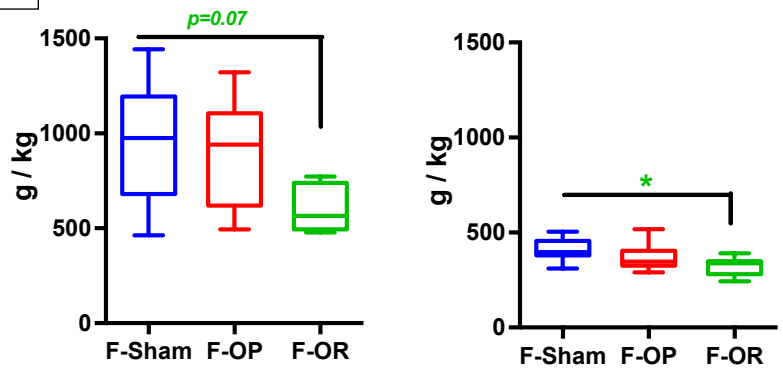
B



C

« goût sucré »

« goût gras »



Femelles

Mâles

Figure 19. Items du comportement alimentaire mesuré chez les descendants des mères OP, OR ou sham en post-sevrage et à l'âge adulte (entre 80 et 100 jours). A, cinétique de prise alimentaire mesurée en post-sevrage entre 22 et 80 jours de vie sous régime chow (*, *P<0,05*, **, *P<0,01*, #, *tendency P<0,1* entre F-OP et F-Sham, *, *P<0,05* entre F-OP et F-OR); B, Mesure de la motivation pour une récompense alimentaire (test en allée droite) en % de succès par rapport au nombre total d'essais (Su= succès ; Fail = échec) ; C, consommations des solutions aux goûts gras (gomme de xanthane, 0,3%) ou sucré (saccharine 0,3%) lors du test des préférences (libre-choix de biberon d'eau ou d'une des solutions).

Les résultats d'analyse du comportement alimentaire en lien avec le microbiote implanté à 21 jours (sevrage) ainsi qu'avec le microbiote adulte ont été publiés dans Frontiers in Microbiology en 2021 (publication #3, Annexe 4, Pocheron *et al.*, 2021, **co-premier auteur avec A-L. Pocheron**).

(b) Recherche de corrélations entre microbiote implanté chez les ratons et la fonction endocrine intestinale

(i) Analyse transcriptomique dans l'iléon et métabolomique

L'analyse ciblée des contenus caecaux ne montrait pas de différence significative de concentration en acides gras à chaîne courte (AGCC) hormis pour la fraction des AGCC mineurs, nous amenant à considérer leur rôle dans les effets observés. De plus, une analyse métabolomique (RMN) réalisée par la plateforme Getplage (INRAE Toulouse) et menée par ma collègue Catherine Michel a permis de fournir des nouvelles pistes de recherche. Je ne présenterai ici que les résultats ouvrant des pistes de recherches intéressantes dans le contexte des interactions entre les CEE et le microbiote intestinal. **Deux étudiants de master2** ont contribué à ce travail (**B. Houssais et K. Seyest**).
L'analyse univariée des concentrations de métabolites des contenus caecaux des ratons à 21 jours n'a fait ressortir que peu de candidats discriminants entre les groupes. Par exemple, la teneur en 5-aminovalérate était significativement diminuée dans le groupe F-OP par rapport à celles du groupe F-OR dans les deux sexes (**Figure 20**). La diminution de ce métabolite intermédiaire du catabolisme des acides aminés est associée à une diminution du métabolisme protéique bactérien et ainsi à une moins bonne récupération d'énergie pour l'hôte (Martin *et al.*, 2010). Précurseur du 5-aminovalerate betaine (5-AVAB), connu pour ses effets positifs sur le neurodéveloppement (Pessa-Morikawa *et al.*, 2022), sa diminution dans le groupe F-OP suggère un potentiel lien avec les défauts de comportements alimentaires observés. Des études *in vitro* sur des neurones embryonnaires de rat sont prévues afin de tester ces métabolites sur le neurodéveloppement.

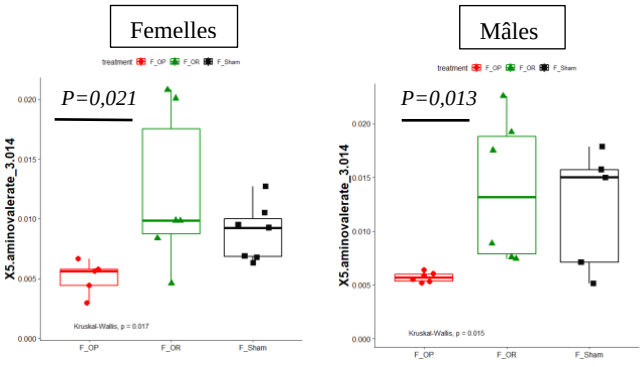


Figure 20. Concentrations en 5-aminovalérate dans les contenus caeco-coliques dans les différents groupes de receveurs. Les différences significatives entre 2 groupes sont indiquées sur le graphe (Kruskall-Wallis et test de Dunn)

L'analyse transcriptomique ciblée dans l'iléon des ratons de 21 jours n'a pas montré de différences significatives dans l'expression des gènes codants pour les hormones intestinales impliquées dans la régulation de la prise alimentaire hormis pour la neurotensine connue pour favoriser l'absorption lipidique et la prise de poids, son expression était diminuée dans le groupe F-OR (**Figure 21**).

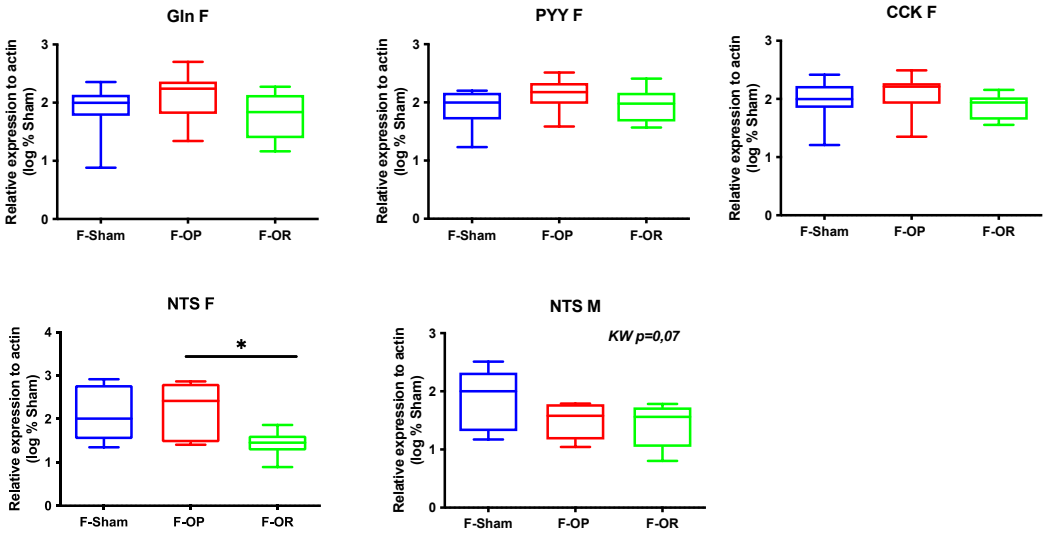


Figure 21. Expression relative dans l'iléon des ratons F-Sham, F-OP et F-OR à 21 jours de quelques gènes codants pour des hormones régulant la prise alimentaire. *, *P<0,05*, test de Kruskall Wallis et test de Dunn pour les comparaisons multiples. Gln, proglucagon ; PYY, peptide YY ; CCK, cholécystokinine, NTS, neurotensine ; M, mâles ; F, Femelles

Sur les différents gènes recherchés caractérisant la fonction endocrine, l'expression de la chromogranine A (ChgA), très fortement exprimée dans les CEE produisant de la sérotonine dans l'intestin distal, était significativement diminuée dans le groupe F-OR (**Figure 22A**) et cette diminution était retrouvée quand les surnageant cæcaux de ces ratons étaient incubés avec une lignée de CEE murine (STC-1) (**Figure 22B**). Une corrélation négative a été retrouvée entre l'expression de ces gènes dans l'iléon et la production de 5-aminovalérate (**Figure 22C**).

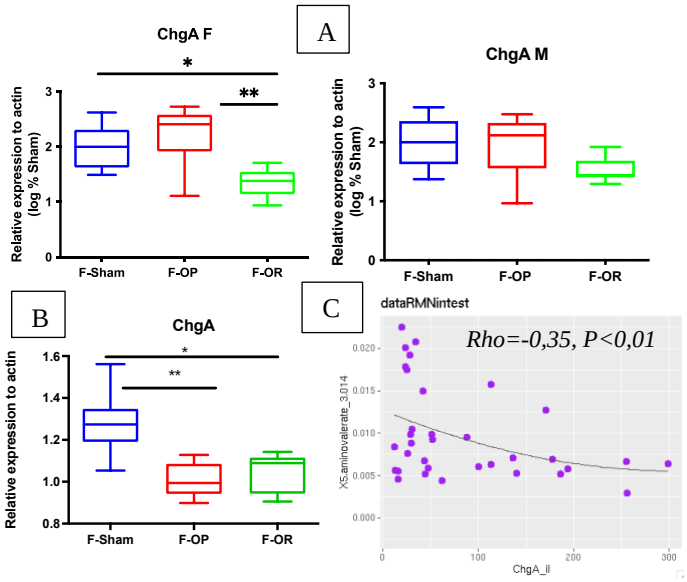
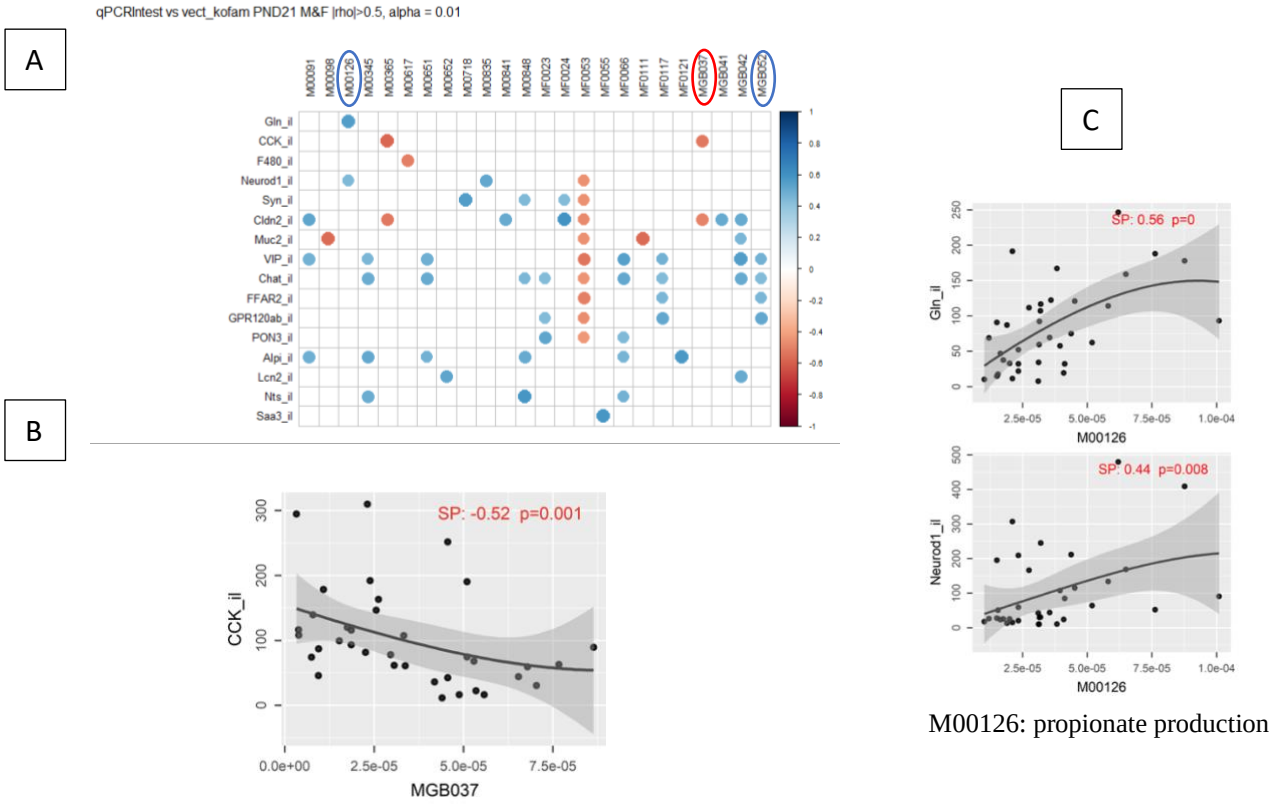


Figure 22. Recherche de corrélation entre l'expression d'un marqueur de CEE et l'activité du microbiote. Expression relative du gène codant pour la chromogranine A (ChgA), A) dans l'iléon des ratons F-Sham, F-OP et F-OR à 21 jours, B) après incubation des cellules STC-1 par les surnageants caecocoliques de ces ratons. *, *P<0,05*, **, *P<0,01*, test de Kruskall Wallis et test de Dunn pour les comparaisons multiples. M, mâles ; F, Femelles, C) corrélation de Spearman entre la

concentration relative de 5-aminovalérate dans les contenus caecocoliques et l'expression de ChgA dans l'iléon.

(ii) Analyse transcriptomique dans l'iléon et fonctions métagénomiques

L'analyse métagénomique (shotgun) a été réalisée dans le cadre d'une collaboration avec Metagenopolis à Jouy-en-Josas (E. Le Chatelier, S. Fromentin). Avec l'aide de Catherine Michel, nous avons recherché des corrélations entre les fonctions métagénomiques prédites par les analyses bio-informatiques et les niveaux d'expressions de gènes de l'iléon et plus particulièrement ceux impliqués dans la différenciation endocrine.



MGB037: γ-Hydroxybutyric acid (GHB) degradation

M00126: propionate production

Figure 23. **Recherche de corrélations entre fonction endocrine intestinale (21 jours) et fonctions métagénomiques.** A) corrélogramme pour des valeurs absolues de Rho >0,5 entre transcriptomique dans l'iléon et espèces métagénomiques enrichies pour des voies de régulations métaboliques (MOO, MFO et MGB, nomenclature du catalogue Métagenopolis pour l'analyse des fonctions métagénomiques), MOO : inclut les grandes voies métaboliques (pathways), MFO inclut le module GMM, Gut Microbiota Metabolism, MGB inclut le module GBM : gut-brain metabolism, B) corrélation de Spearman entre l'expression du gène codant pour la CCK et la voie de dégradation du GHB, C) corrélation de Spearman entre l'expression du gène codant pour le proglucagon (Gln) et Neurod1 avec la voie de production du propionate.

De façon intéressante, l'expression de la CCK dans l'iléon était négativement corrélée à la voie de dégradation du GHB, un acide gras à chaîne courte dérivé du GABA (**Figure 23A&B**) alors

que l'expression du proglucagon était positivement corrélée à la production de propionate (**Figure 23A&C**). De même, l'expression des récepteurs aux AGCL (GRP120=FFAR4) ou AGCC (GPR 43 =FFAR2) était positivement corrélée à une voie de synthèse du butyrate (**Figure 24**). Ces observations n'expliquent à ce stade aucun lien causal mais ouvrent néanmoins des nouvelles pistes de recherche pour décrire et comprendre les interactions entre les CEE et le microbiote intestinal.

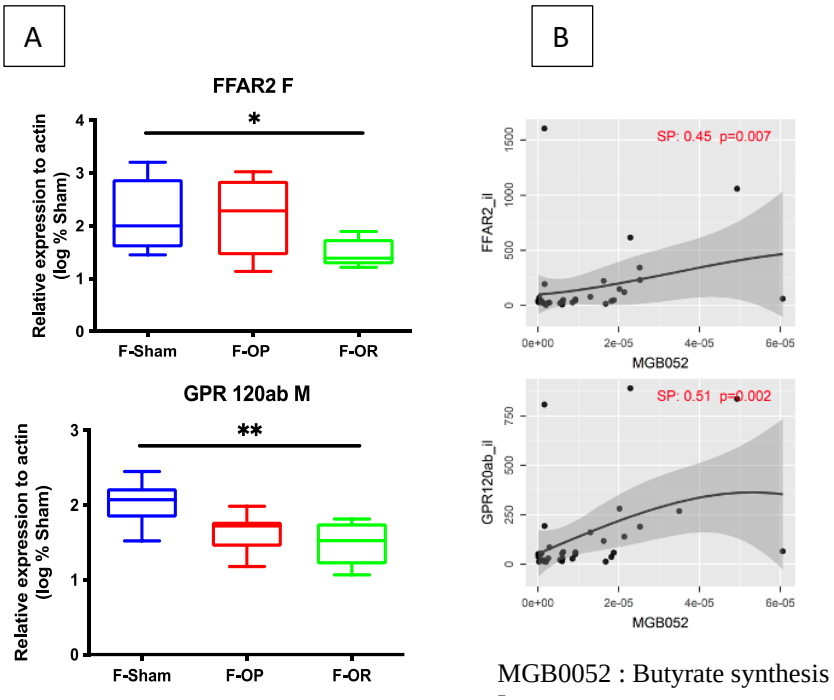


Figure 24. Expression des récepteurs aux AG et fonction métagénomique à 21 jours. A) Expression relative des gènes codant pour les récepteurs aux acides gras dans l'iléon des ratons, *, *P<0,05*, **, *P<0,01*, test de Kruskall Wallis et test de Dunn pour les comparaisons multiples. M, mâles ; F, Femelles, B) corrélation de Spearman entre l'expression de ces gènes et la voie de synthèse du butyrate.

(iii) Transcriptomique des CEE STC-1 et fonctions métagénomiques

Des recherches de corrélation entre l'expression de gènes endocrines mesurées après incubation de la lignée murine STC-1 avec les surnageants caecaux des ratons à 21 jours ont montré des relations négatives entre l'expression du proglucagon et une des voies de synthèse du glutamate et de l'acétate (**Figures 25A&B**). L'expression du PYY était corrélée à celle la synthèse de dopamine (**Figure 25A&C**) et inversement corrélée à la voie impliquant ClpB, protéine bactérienne chaperone ayant un motif α-MSH anorexigène. Les effets anorexigèniques de ClpB impliqueraient la production de GLP-1 et de PYY par les CEE-L (Breton *et al.*, 2016). Ces pistes sont particulièrement intéressantes car nos résultats montrent bien que les défauts de régulation de comportement alimentaire à l'âge adulte ne sont pas dus au microbiote intestinal dont la composition n'est plus significativement différente entre nos groupes à ce stade. C'est donc bien au moment où les microbiotes étaient différents, *i.e.* à 21 jours, que les effets sur le neurodéveloppement ont pu se produire.

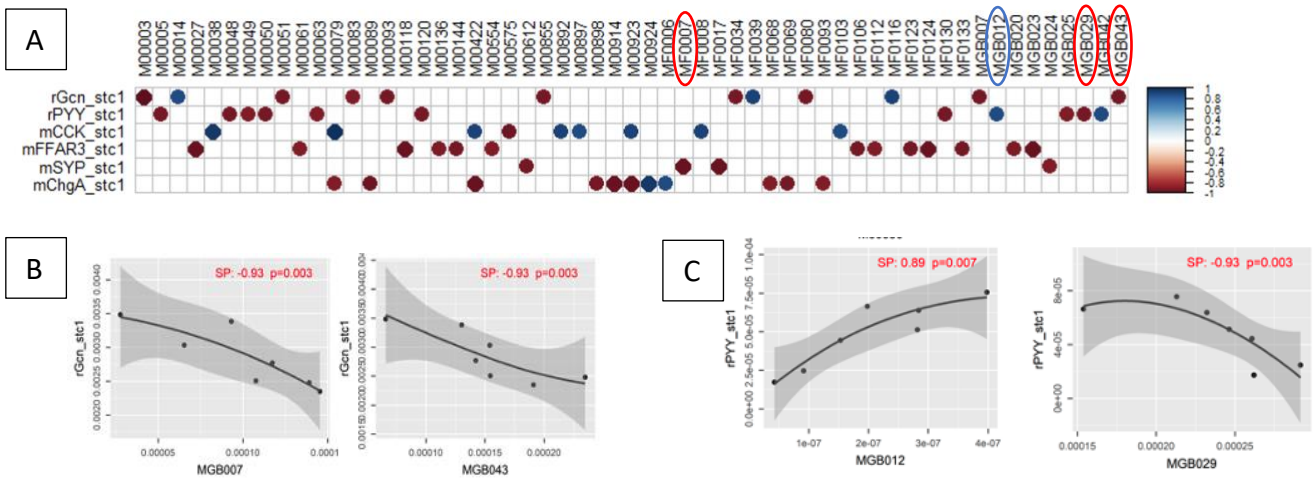


Figure 25. Fonction endocrine *in vitro* (STC-1) et fonction métagénomique à 21 jours. A) corrélogramme pour des valeurs absolues de Rho >0,5 (P<0,01) entre transcriptomique des cellules STC-1 et espèces métagénomiques enrichies pour des voies de régulations métaboliques (catalogue Métagenopolis, cf figure 19), B) corrélation de Spearman entre l'expression du gène codant pour le proglucagon et une voie de synthèse du glutamate (MGB007) et de l'acétate (MGB043), C) corrélation de Spearman entre l'expression du gène codant pour le PYY avec la voie de production de la dopamine (MGB012) et de ClpB (MGB029) par le microbiote intestinal.

Les résultats obtenus dans ce projet devraient être valorisés prochainement dans un article mettant en regard les données corrélant composition et fonction du microbiote intestinal à l'échelle métagénomique avec la fonction endocrine intestinale et celles obtenues de façon parallèle dans le cerveau (collaboration avec P. Parnet). En effet, si le microbiote intestinal précoce, transmis par la mère, a eu un effet en début de vie sur le neurodéveloppement de la descendance, un des mécanismes sous-jacents pourrait être par les interactions des métabolites bactériens avec l'épithélium intestinal et notamment les CEE qui, comme décrit précédemment, sont les sentinelles de l'axe intestin-cerveau. Il est tout à fait envisageable que ces cellules produisent, en réponse à l'activité du microbiote intestinal, des peptides, des amines ou encore des neuromédiateurs pouvant avoir des effets neurotropes. La proximité de l'épithélium intestinal avec le système entérique nous amène à considérer les effets de certains métabolites bactériens identifiés dans les analyses de corrélations sur les interactions CEE-SNE.

C. FONCTION INCRÉTINE DE LA DESCENDANCE ISSUE D'UN DIABÈTE GESTATIONNEL

1. POSITIONNEMENT DU PROJET

Comme présenté en introduction, j'ai activement participé à la rédaction du projet ANR GDM-MILK porté par ma collègue Marie-Cécile Alexandre-Gouabau. En 2020, je souhaitais développer un projet au laboratoire pour étudier les effets des composants du lait maternel sur le développement et la maturation de l'intestin, et plus particulièrement sa fonction endocrine. L'objectif était d'identifier des composés bioactifs ayant des effets bénéfiques sur la lignée endocrine afin de les utiliser à des fins préventives dans des situations de programmation nutritionnelle ou métabolique. De son côté, M.C. Alexandre-Gouabau avait pour projet de mener une étude sur le devenir métabolique d'une descendance née de mères hyperglycémiques

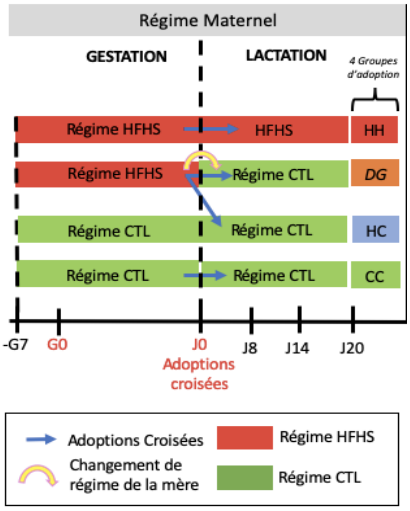
pendant la gestation et d'investiguer la fenêtre de lactation comme levier pour moduler le risque d'apparition de diabète de type 2 plus tard dans la vie. Des données cliniques préliminaires (cohorte canadienne DEPART) semblaient en effet indiquer une adaptation de la composition du lait chez des mères atteintes de diabète gestationnel (DG) et des données publiées montraient une protection de l'enfant allaité par une mère DG (Mayer-Davis *et al.*, 2008). Le DG est un exemple particulièrement préoccupant de programmation métabolique puisqu'il concerne une mère sur six dans le monde (Saeedi P *et al.*, International Diabetes Federation, 2019) et contribue à alimenter l'épidémie de DT2, notamment chez les jeunes (Lowe *et al.*, 2019; Eletri *et* Mitanchez, 2022). Nous avons ainsi construit le projet GDM-Milk qui a reçu un financement de l'ANR en 2022. Dans ce projet, je développe ma question de l'effet des composants du lait maternel sur la fonction endocrine intestinale par plusieurs approches :

- *In vivo*, dans un modèle rongeur de DG, des adoptions croisées permettent d'étudier les effets de la composition d'un lait DG *vs* non DG sur la densité de CEE et notamment celles produisant du GLP-1, hormone incrétine, dans l'intestin des descendants au cours de leur développement post-natal et jusqu'à l'âge adulte
- *In vitro*, par un criblage des molécules identifiées dans la cohorte DEPART comme étant modulées par le DG sur leur capacité à induire une synthèse et/ou une sécrétion de GLP-1 sur une lignée de CEE humaine (NCI-h716)

2. RÉSULTATS PRÉLIMINAIRES

a) In vivo

Le modèle *in vivo* a été mis en place et validé dans le cadre de la **thèse de Paul Bobin que je co-encadre** avec M.C. Alexandre-Gouabau (financement INRAE-région pays de La Loire). Nous avons choisi un modèle nutritionnel de DG dans lequel les rates reçoivent un régime hyperlipidique et riche en sucre (HFHS) une semaine avant l'accouplement et durant les 3 semaines de lactation. Les groupes expérimentaux sont représentés sur la **figure 26**.



• Groupe HH : Ratons nés de mères hyperglycémiques et allaités par des mères sous régime hyperglycémiant pendant la gestation et la lactation.

• Groupe DG : Ratons nés de mères hyperglycémiques et allaités par des mères sous régime hyperglycémiant pendant la gestation et redevenues normo-glycémiques pendant la lactation par adoption d'un régime standard après la mise bas.

• Groupe HC : Ratons nés de mères hyperglycémiques pendant la gestation et allaités par des mères sous régime contrôle normo-glycémiques pendant la gestation et la lactation.

• Groupe CC : Ratons nés de mères normo-glycémiques et allaités par des mères sous régime contrôle normo-glycémiques pendant la gestation et la lactation.

Figure 26. Schéma expérimental du modèle de DG chez le rat montrant les différents groupes expérimentaux obtenus par adoptions croisées. CTL, control (chow); HFHS, high-fat high-sucrose; G, gestation.

Le modèle a été validé dans la thèse de Paul Bobin. Les mères HFHS présentaient bien une

hyperglycémie pendant la gestation, qui disparaissait pendant la lactation. La descendance mâle du groupe HC à l'âge adulte placée sous régime hypercalorique montrait une baisse de sa capacité à sécréter de l'insuline (test OGTT) et les femelles du même groupe une sensibilité à l'insuline moindre (clamps euglycémiques). L'analyse des trajectoires métaboliques sur les descendants est en cours.

Dans le cadre du **master2 de Marie Michelland (2023**), nous avons pu mettre en évidence par des analyses immunohistochimiques que la densité de CEE-L (ratio CEE-L/CEE totales) était significativement plus faible dans les iléons des ratons mâles à 21 jours dans les groupes DG, HC et HH par rapport à ceux du groupe CC (**Figure 27**).

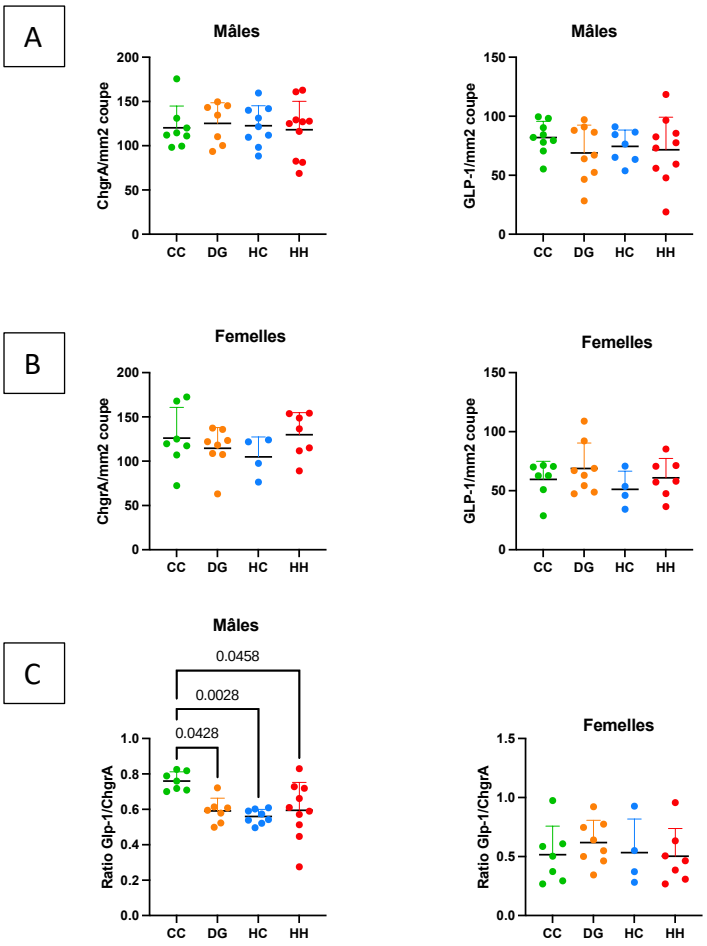


Figure 27. **Densités des CEE totales (ChgrA) et CEE-L (Glp-1) mesurées dans l'iléon des ratons (21 jours) dans les différents groupes d'allaitement**. A) Mâles B) Femelles C) ratio Glp-1/ChgrA. Test de Kruskall-Wallis et test de Dunnet pour les comparaisons multiples.

Ces résultats montrent pour la première fois l'effet du régime maternel pendant la gestation sur les CEE-L au sevrage et soulignent l'importance de la période de gestation. En effet, le régime maternel (CTL ou HFHS) pendant la lactation était sans effet.

Les effets d'une alimentation riche en graisses sur la densité des CEE-L ont été bien documentés chez l'adulte mais les données sont contradictoires. Chez la souris par exemple, une exposition pendant deux semaines à un régime riche en graisses (60% de lipides) diminuait l'expression du gène du proglucagon dans le côlon, associée à une réduction des CEE-L (Richards *et al.*,

2016). Un régime moins riche en lipides (34%) mais donné pendant 8 ou 16 semaines, rendant les souris obèses, avait un effet inverse en augmentant la densité des CEE productrices de GLP-1 (Aranias *et al.*, 2015 ; Dusaulcy *et al.*, 2016). Chez l'homme obèse consommant plus de 30% de lipides dans son alimentation, la densité de CEE-L dans le jéjunum était également significativement augmentée par rapport à des patients obèses consommant des quantités inférieures de lipides (Aranias *et al.*, 2015). Il apparaît que la durée du régime obésogène mais également la mise en place d'une intolérance au glucose (Dusaulcy *et al.*, 2016) contribuent à augmenter la densité des CEE productrices de GLP-1. Dans notre étude, la composition du lait maternel DG, HC ou HH n'a pas modifié la densité de ces cellules dans l'iléon au sevrage suggérant que la diminution de la densité des CEE-L est due à l'hyperglycémie maternelle induite par le régime HFHS *in utero*. Une analyse transcriptomique de ces CEE est prévue afin de rechercher les effets de cette programmation sur les facteurs de transcription impliqués dans la différenciation des CEE-L pendant la période périnatale. Comme évoqué plus haut, je mise sur la possibilité d'extraire les ARN totaux de ces cellules après microdissection laser (plateforme Micropicell). D'autre part, des analyses à temps plus tardifs sont en cours afin de définir si cette programmation par le régime HFHS *in utero* persiste en post-sevrage et d'interroger comment ces CEE s'adaptent à un challenge hypercalorique plus tard dans la vie. Les différences observées entre les deux sexes sont difficiles à expliquer. Le suivi métabolique de la descendance mâle et femelles effectué dans la thèse de P. Bobin montre en effet que seuls les mâles du groupe HC ont une capacité à sécréter de l'insuline (test OGTT) réduite sous challenge hypercalorique.

b) In vitro

Les analyses de la composition du lait des mères de la cohorte DEPART faisaient apparaître des variations quantitatives de certains composés lipidiques complexes tels que des sphingolipides ou des céramides ainsi que certains acides aminés. Nous avons donc entrepris avec l'aide de Pierre de Coppet, ingénieur d'études au laboratoire, d'étudier les effets de ces composés sur la synthèse et la sécrétion de GLP-1 sur la lignée endocrine NCI-h716. Les résultats concernant les acides aminés ont confirmé l'effet sécrétagogue sur le GLP-1 de la glutamine (**Figure 28**) comme déjà publié sur la lignée GLUTag (modèle de CEE-L, Reimann *et al.*, 2004, Tolhurst *et al.*, 2011, Nakamura *et al.*, 2020), ainsi que de la valine et de l'arginine dans un modèle de perfusion duodénale chez le rat (Modvig *et al.*, 2021). L'effet de la glycine n'atteint pas la significativité dans notre étude contrairement à Gameiro *et al.* (2005) sur la lignée GLUTag.

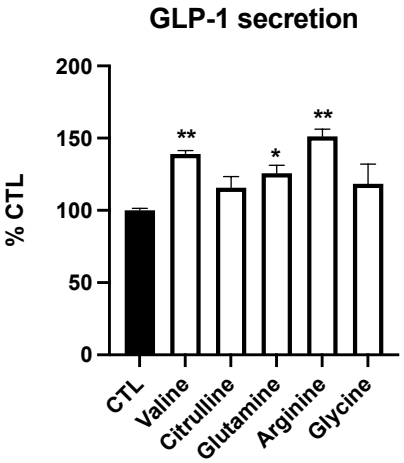


Figure 28. Sécrétion de GLP-1 exprimée en % du témoin (CTL) par les NCI-h716 après 2h d'incubation en présence d'acides aminés (10 mM). *, *P<0,05* ; **, *P<0,01*, différences significatives du groupe CTL. T-test.

Concernant les lipides complexes, les données préliminaires obtenues dans la cadre du master2

de Marie Michelland sont à confirmer par de nouvelles manipulations en raison d'une grande variabilité des résultats, comme illustré ci-dessous (**Figure 29**) pour le glucosylcéramide d18 :1/24 :1 ou la sphingomyéline d18 : 1/18/0.

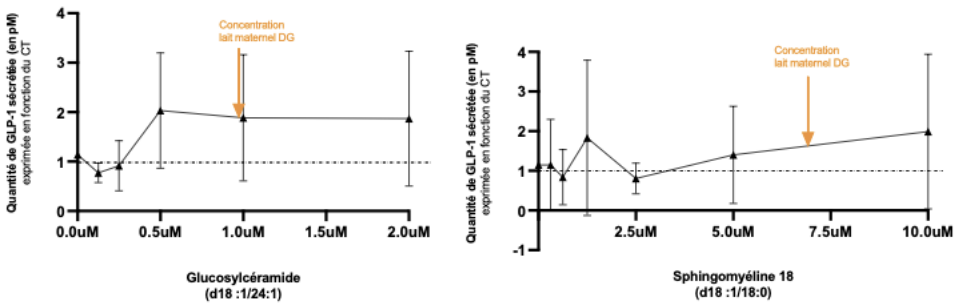


Figure 29 : Sécrétion de GLP-1 (ELISA) par la lignée cellulaire NCI-H716, exprimée en fonction du témoin (CT), en présence des différents sphingolipides.

La mise évidence d'un effet sur la sécrétion de GLP-1 d'un ou plusieurs composés bioactifs du lait de mères DG a pour objectif final de valider leur effet *in vivo* en les supplémentant à des ratons nés de mères DG et allaités par des mères non DG. Le suivi métabolique de cette descendance, en comparaison avec des ratons nés de mères DG et allaités par des mères DG devrait permettre de confirmer ou invalider l'hypothèse d'un effet « protecteur » du lait de mères DG par l'adaptation de sa composition à l'hyperglycémie pendant la gestation.

Les expérimentations menées sur le modèle *in vivo* de DG avec adoptions croisées vont produire de nouvelles connaissances sur les effets d'un régime maternel hypercalorique (conduisant à une hyperglycémie) pendant la gestation et/ou lactation sur le lignage entéro-endocrine de la descendance.

Dans le cadre de sa thèse, Paul Bobin doit effectuer un séjour de 3 mois à l'international (programme de l'EIR-A, Agreenium). Il sera accueilli au MRC Institute of Metabolic Science de Cambridge, dans l'équipe de F. Gribble et F. Reimann, spécialistes de la CEE-L. Au-delà de la formation de Paul et de la création de son réseau scientifique, cette nouvelle collaboration est très stimulante pour ma question scientifique et pourrait déboucher sur des projets de plus grande envergure à l'avenir.

IV. PERSPECTIVES ET NOUVEAU PROJET

Dans la période à venir, je vais m'attacher à poursuivre mon travail sur ma question centrale : **les CEE sont-elles des cibles d'un environnement maternel défavorable (nutritionnel ou métabolique) pouvant relayer des effets sur la régulation du comportement alimentaire et de l'homéostasie glucidique adulte ?** Mon projet continuera à porter sur le développement, le renouvellement à partir des cellules souches adultes et la maturation des CEE suite à une malnutrition ou un déséquilibre métabolique maternels. La composante microbiote et sa modulation pendant la période néonatale continueront d'être explorées. Cette question sera adressée dans la continuité de résultats obtenus dans la précédente période (projets Olygose, Mamiprooffi, PARIMAD, GDM-MILK). Je compte également développer un nouveau projet sur le rôle de la nutrition périnatale et du microbiote sur la protéine Tau exprimée dans les CEE suite à des résultats très originaux obtenus dans le cadre d'une collaboration avec l'UMR Tens (P. Derkinderen). Mes perspectives se déclinent ainsi selon les objectifs suivants :

1. MONTRER COMMENT LES MÉTABOLITES BACTÉRIENS FAVORISENT LA DENSITÉ DES CELLULES L DANS L'ILÉON MAIS PAS DANS LE CÔLON (suite projet Olygose).

Les effets « segments spécifiques » des prébiotiques que nous avons observés dans l'intestin des ratons supplémentés pendant la période post-natale ouvrent de nouvelles pistes de recherche sur la régulation du lignage endocrine par le microbiote intestinal. Ils interrogent le concept de plasticité « régionale » des CEE matures proposé par l'équipe de H. Clevers (Beumer *et* Clevers, 2021). Si le caractère multihormonal des précurseurs de CEE a été démontré chez plusieurs espèces incluant le rat (Svendsen *et al.,* 2015), la plasticité hormonale des CEE au stade mature est une hypothèse très récente mise en avant par des études transcriptomiques « single cell » au cours de la différenciation et maturation des CEE à partir de leur progéniteur Ngn3+ (Gehart *et al.,* 2019). Cette étude montre par exemple que dans 70% des cellules L et I, la corrélation entre l'expression de leurs hormones est très forte. Ainsi, selon la nature des signaux le long de l'axe crypto-villositaire, une CEE L/I pourra sécréter du GLP-1 (cellule L) ou de la CCK (cellule I) (Beumer *et al.,* 2018 ; Gehart *et al.*, 2019). Il est donc probable que le contenu de la lumière intestinale et notamment les métabolites bactériens, puissent avoir un effet sur ces processus de maturation et de plasticité.

A la suite du projet Olygose, notre hypothèse était que les métabolites produits par le microbiote encore en cours d'implantation du raton (*i.e.* dénué de capacité butyrogène) pourraient agir sur la plasticité de CEE en cours de maturation à produire du GLP-1. Pour répondre à cette question, nous souhaitions développer la culture d'organoïdes à partir de cryptes intestinales de rats pCCK-GFP. Ce modèle aurait permis de suivre *in vitro* l'évolution de la fluorescence comme marqueur de la plasticité des CEE en cours de maturation vers la production de CCK (GFP+) ou de GLP-1en présence des surnageants iléaux ou caecaux des ratons ayant reçu des OS ou les métabolites bactériens significativement augmentés (acétate, lactate).
A l'aide d'un financement obtenu auprès de la fondation SantéDige du CHU de Nantes, nous avions démarré en 2021 avec P. de Coppet, la mise au point de la production d'entéroïdes à partir de cryptes iléales et coliques de jeunes rats. Malgré de nombreux essais (milieu conditionné L-WRN pour organoïdes de souris (collaboration Claire Cherbuy, INRAE Micalis, Jouy-en Josas), milieu commercial pour organoïdes humains (Stem Cell), essais avec des facteurs de croissance spécifiques, etc.), nous ne sommes pas parvenus à obtenir des organoïdes chez cette espèce. Ce projet est donc à l'arrêt actuellement. La possibilité de mener cette étude sur des organoïdes de souris est envisagée sous réserve d'un financement permettant soit d'acquérir la technique auprès d'un laboratoire partenaire ou soit de sous-traiter la manipulation sur une plateforme nationale.

2. MONTRER COMMENT LE MICROBIOTE NÉONATAL IMPACTE LE NEURODÉVELOPPEMENT EN ÉTUDIANT LES INTERACTIONS ENTRE MÉTABOLITES BACTÉRIENS ET CEE (poursuite du projet Mamiprooffi)

La poursuite de ce projet s'inscrit dans l'étude des mécanismes visant à comprendre comment le microbiote, et plus particulièrement les espèces bactériennes qui ont été identifiées comme pouvant contribuer à « un phénotype obèse » pourraient moduler le développement du système neuroendocrine et le cerveau. En effet, le microbiote intestinal produit des métabolites de type neurotransmetteurs qui peuvent agir directement sur le neurodéveloppement. Mais une autre voie possible implique les interactions microbiote-CEE elles-mêmes en contact avec le système nerveux entérique et le nerf vague qui relaie la signalisation endocrine au cerveau (**Figure 30**). Des expériences sur lignées immortalisées de CEE (GLUTag, STC-1, NCI-h716) en co-culture avec des neurones extraits de ganglion plexiforme (afférences vagales) ou des neurones embryonnaires de rat ou de la glie entérique (collaboration avec l'UMR Tens) seront réalisées en les incubant avec les surnageants caeco-coliques des ratons ayant reçu les souches des bactéries candidates. Des marqueurs de CEE et d'activation gliale ou permettant de dénombrer et de caractériser les neurones seront utilisés.
Ce travail sera réalisé à la suite de l'étude menée actuellement par C. Heberden (INRAE Micalis, Jouy-en Josas, partenaire de l'ANR Mamiprooffi) qui teste actuellement l'effet de ces bactéries candidates sur le comportement alimentaire de ratons en isolateurs.

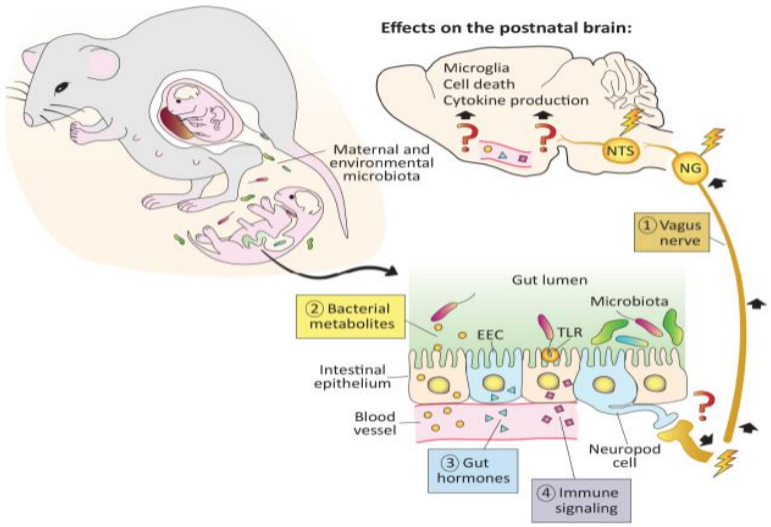


Figure 30. Voies potentielles d'action du microbiote néonatal sur le neurodéveloppement (d'après Gars *et al.*, 2021)

3. UN NOUVEAU PROJET AUTOUR DES CEE : L'EXPRESSION DES PROTÉINES TAU ET α-SYN DANS LES CEE HUMAINES CONTRIBUE-T'ELLE À LA MALADIE DE PARKINSON ? QUEL EST LE RÔLE DE LA NUTRITION PERINATALE DANS LE RISQUE DE TAUOPATHIE?

Financement ANR Enteroendopark (Pascal Derkinderen, UMR 1235 Tens, INSERM-NU)

Les protéines Tau et α-syn sont des acteurs connus des lésions caractérisant les maladies neurodégénératives. Les protéinopathies telles que la maladie d'Alzheimer (AD) ou de Parkinson (PD) partagent les mêmes caractéristiques : perte neuronale, agrégat de protéines Tau (avec des neurofibrilles) ou α-synucléine (α-syn) (corps de Lewy) phosphorylées et diffusion de la pathologie qui serait liée au passage de ces protéines pathologiques de neurones à neurones (Duyckaerts *et al.,* 2019). Si l'hypothèse d'une origine intestinale de la maladie (Braak *et al.*, 2003) est encore débattue (Rietdijk *et al.*, 2017 ; Lionnet *et al.,* 2018 ; Arotcarena *et al.,* 2020), elle n'est pas complètement abandonnée et des études d'imageries *in vivo* chez l'homme proposent une nouvelle hypothèse selon laquelle 50% des patients auraient une PD d'origine périphérique (body-first PD) et 50% d'origine centrale (brain-first PD) (Borghammer *et* Van Der Berge, 2019). De plus, des troubles de transit colique (constipation) sont fréquemment (70% des cas) rapportés chez les patients PD (Knudsen *et al.,* 2017).

Dans le cadre d'un projet collaboratif avec Pascal Derkinderen, neurologue spécialisé dans la maladie de Parkinson qui étudie la contribution intestinale (SNE) dans les maladies neurodégénératives, nous avons mis en évidence l'expression de Tau dans les CEE du côlon humain (publication#1, Annexe 5, Chapelet *et al.,* 2023), comme cela avait déjà été démontré précédemment pour α-syn (Chandra *et al.*, 2017). Le fait que ces protéines exprimées dans les neurones le soient également dans des cellules épithéliales intestinales qui elles-mêmes possèdent un certain nombre de caractéristiques neurales (excitabilité, production de neuropeptides, expression de récepteurs présynaptiques, neuropode…) nous amènent à considérer leur rôle dans la transmission de la maladie (tauopathie ou α-synucléinopathie) depuis la sphère digestive. Notre étude démontre également que Tau est phosphorylée dans les CEE humaines, et dans une lignée murine de CEE-L (GLUTag), un traitement au propionate et au butyrate diminuait la phosphorylation de Tau (Chapelet *et al.,* 2023). La signification de cette phosphorylation de Tau dans les CEE reste à découvrir mais Tau pourrait, comme cela a été montré dans les cellules ß-pancréatiques (Wijesekara *et al.,* 2018), jouer un rôle clef dans la régulation de la production d'hormones.

Nos travaux montrant l'expression et la phosphorylation de Tau dans les CEE ainsi que les travaux publiés sur α-syn ont conduit à l'obtention d'une ANR visant à comprendre comment l'expression ces deux protéines dans les CEE pourrait contribuer à la maladie de Parkinson.

Le projet ANR Enteroendopark décline plusieurs objectifs :

1. Etudier les effets des AGCC sur la régulation de l'expression, la phosphorylation, l'agrégation et la sécrétion de Tau et α-syn dans des lignées de CEE humaines
2. Etudier les effets de ces protéines sécrétées sur le SNE (neurones et glie) et les afférences vagales à proximité
3. Comprendre le rôle de Tau et d'α-syn sur les fonctions des CEE
4. Rechercher des modifications de Tau et α-syn dans les CEE de patients parkinsoniens

Pour répondre à ces objectifs, en collaboration avec l'UMR Tens, nous utiliserons des modèles cellulaires simples (lignées de CEE humaines ou murines) ou plus complexes (co-cultures CEE

neurones) ainsi que des biopsies coliques et des prélèvements digestifs coliques 'full thickness' de patients parkinsoniens (Gelpi *et al.,* 2014).
Je prendrai en charge l'objectif 3 en réalisant des transfections de formes humaines de Tau et d'α-syn dans les lignées NCI-h716 et GLUTag pour mesurer l'impact de leur sur-expression sur la fonction principale des CEE, synthèse (RTqPCR) et sécrétion (ELISA) d'hormone (GLP-1) en réponse à la détection de métabolites bactériens (AGCC). Le flux calcique intracellulaire sera mesuré à l'aide du système GCaMP. La capacité des CEE à faire synapse avec les afférences vagales et/ou le SNE sera mesurée par électrophysiologie sur des neurones primaires du SNE ou du ganglion plexiforme (collaboration V. Paillé, PhAN) cultivées en présence de CEE NCI h-716 sur-exprimant Tau et/ou α-syn.
Pour atteindre l'objectif 4, nous souhaitons accéder au transcriptome des CEE coliques de patients parkinsoniens par une approche de biologie spatiale (technologie Merscope, Vizgen) Cette nouvelle technologie permettra de mesurer spécifiquement l'expression de Tau et α-syn dans les CEE coliques des patients et d'obtenir un large panel des différentes voies de régulation conduisant à l'agrégation de Tau et α-syn dans ces cellules.

Le financement du projet Enteroendopark représente une opportunité d'interroger le rôle des CEE dans les maladies neurodégénératives. Au-delà des enjeux de ce projet - biomarqueurs précoces de la maladie, production de connaissances sur l'axe intestin-cerveau dans la maladie de Parkinson - je souhaite profiter de cette dynamique pour introduire de la périnatalité dans ces questions.

Périnatalité, Tau dans les CEE et tauopathie plus tard ans la vie

Comme défini précédemment, selon le concept de la DOHaD, un environnement périnatal altéré par le stress maternel, une exposition à des toxiques ou encore par une malnutrition a des conséquences à long terme et peut « programmer » chez la descendance adulte des maladies chroniques, impliquant des dysfonctions métaboliques mais aussi à des désordres cognitifs. Or chez les patients atteints de maladies neurodégénératives telles que la maladie d'Alzheimer, à la fois les troubles cognitifs et métaboliques sont présents. Ces troubles se développent au cours du vieillissement mais pourraient également avoir une origine périnatale (revue de Gauvrit *et al.,* 2022). La littérature sur les effets à long-terme de l'environnement précoce sur le développement des maladies neurodégénératives et notamment sur les lésions caractéristiques de l'AD- la formation des plaques amyloïde et l'agrégation de Tau- est relativement abondante. Celle sur les effets d'une malnutrition maternelle l'est moins ; les conclusions ne sont toujours concordantes et dépendent de la fenêtre d'exposition. Dans un modèle de souris AD triple transgénique[1], Martin *et al.* ont montré qu'une exposition pendant la gestation et la lactation exacerbait la tauopathie dans l'hippocampe, pouvant ainsi sensibiliser au développement d'une AD (Martin *et al.,* 2014). En revanche, chez des souris WT, une exposition au même régime mais restreinte à la gestation avait des effets plutôt bénéfiques à long terme sur la cognition mesurée à 18 mois (*via* une diminution de l'expression de Tau et de ses formes pathologiques, améliorant ainsi l'intégrité synaptique dans l'hippocampe) (Di Meco *et al*., 2019). Un relais mécanistique pouvant expliquer ces effets est le microbiote intestinal. Le régime maternel pendant la période périnatale modifie la composition des microbiotes (intestinal, vaginal, lait) de la mère qui le transmet au petit (revue Browne *et al.,* 2022). Ainsi il a pu être mis en évidence chez la souris qu'un régime maternel hyper-lipidique altérait la

[1] Modèle de souris transgénique exprimant des gènes humains portant des mutations connues dans la maladie d'Alzhzeimer (APP_{Swe}, $PS1_{M146}$ and tau_{P301L}).

mémoire à court-terme chez la descendance et que cet effet pouvait en partie être lié à une dysbiose (Sanguinetti *et al.,* 2019). De façon intéressante, un régime riche en fibres rétablissait les désordres cognitifs observés chez la descendance née de mères obèses (Liu *et al.,* 2021) et ces effets positifs étaient dus au changement de microbiote et des AGCC produits. D'autres études ont montré les effets régulateurs des AGCC sur le neurodéveloppement (Erny *et al.,* 2015) soutenant un rôle majeur de l'axe microbiote-intestin-cerveau dans les fonctions du système nerveux central. Je souhaiterais ainsi à plus long terme étudier l'hypothèse suivante : « La modulation de l'expression et de la phosphorylation de Tau dans les CEE par le microbiote (ou malnutrition maternelle) en période périnatale favoriserait l'évolution d'une tauopathie plus tard dans la vie ».

a. Preuves de concept

- *In vivo*

Pour démontrer les effets précoces et à long-terme de l'environnement périnatal sur l'expression et la phosphorylation de Tau dans les CEE nous utiliserons les prélèvements intestinaux obtenus dans nos différents modèles de programmation.

1. Malnutrition maternelle :
    - Descendants nés de mères restreintes à 8% de protéines pendant la gestation et/ou la lactation
    - Descendants nés de mères en hyperglycémie maternelle pendant la gestation
2. Modulation du microbiote néonatal
    - Ratons nouveau-nés supplémentés par des oligosaccharides prébiotiques (chez qui densité CEE et CEE-L est fortement augmentée, Le Dréan *et al.,* 2019)
    - Ratons nouveau-nés ayant reçu des microbiotes maternels (vaginal, intestinal et issus du lait) de compositions différentes

L'expression de Tau et de sa phosphorylation seront d'abord analysées globalement par Western-Blot sur les parois des iléons ou côlons prélevés dans ces modèles au stade précoce (21 jours) et plus tard dans la vie (entre 150 et 200 jours). Une analyse plus spécifique sur les CEE sera réalisée par immunofluorescence. L'expression des marqueurs de tau total (MAPT) et de ses isoformes sera mesurée par RT-qPCR. Un double marquage caspase-3/Chgra en faveur d'un clivage de Tau et de son état favorable à son agrégation pourra également être réalisé.

Les échantillons intestinaux dont nous disposons au laboratoire sont prélevés sur des animaux âgés de 200-240 jours maximum. Il est possible que cet âge soit insuffisant pour observer des modifications liées au vieillissement de Tau. Une nouvelle expérimentation sur l'un ou l'autre de ces modèles de programmation pourra alors être conduite en maintenant les animaux à un âge plus avancé (autour de 9-10 mois).

- *In vitro*

Nous disposons pour la plupart de ces modèles des surnageants caeco-coliques (métabolites bactériens) que nous proposons d'incuber sur des lignées de CEE murines (GLUTag) ou humaines (NCI-h716) afin de mesurer les effets sur Tau et phospho Tau par Western-Blot. Des phosphorylations d'épitopes spécifiques et des formes tronquées en carboxy-terminal sont caractéristiques des formes pathogéniques de Tau. Ainsi, MC1, forme pathogénique clivée sur

le site Asp421 de Tau par la caspase-3, permet l'agrégation de Tau en « neurofibrillary tangles ». Des anticorps spécifiques de ces formes phosphorylées sont disponibles dans le commerce et déjà utilisée en Western-Blot à l'UMR Tens (Prigent *et al.,* 2020).
Des métabolites déjà identifiés comme significativement augmentés dans nos modèles de programmation comme par exemple le 5-aminovalérate seront incubés en présence de CEE NCI-h716 et GLUTag transfectées par des formes pathogéniques de Tau (P301L par ex) afin de rechercher des effets favorisants ou prévenant la tauopathie.

b. Validation dans des modèles murins de tauopathie

Selon le niveau de preuve obtenu précédemment, j'envisage de poursuivre l'étude sur des modèles murins de l'AD qui seront soumis à un modèle de programmation nutritionnelle en période périnatale, par exemple un régime hypercalorique pendant la gestation et/ou la lactation afin de récupérer les intestins à différents stades de la vie pour analyser la lignée endocrine. La souris THY-Tau22, forme humaine mutée, présente des enchevêtrements neurofibrillaires caractéristiques de la tauopathie. Ce modèle utilisé par l'équipe de D. Vieau N. Sergeant à Lille montre des altérations cognitives à 6 mois (mémoire spatiale). Cette équipe a montré qu'un régime riche en lipides pendant la lactation (58%) augmentait la phosphorylation de Tau dans l'hippocampe, amplifiant ainsi la tauopathie (résultats non publiés présentés à la Journée Scientifique de NU en juin 2023). Il serait intéressant d'étudier la phosphorylation de tau dans les CEE de ce modèle et de rechercher si l'effet du régime hyper-calorique pendant la lactation affecte- d'abord, de façon concomitante ou après-, les CEE et les neurones hippocampiques.

Ce nouveau projet devrait permettre de déterminer la contribution des CEE et de l'axe intestin-cerveau dans la physiopathologie des tauopathies en collaboration avec l'UMR Tens. Nous obtiendrons des données sur le rôle de la nutrition périnatale sur l'expression et la phosphorylation de Tau et de ses formes pathologiques dans les CEE, alimentant ou limitant une prédisposition à développer la maladie plus tard dans la vie. L'expression de Tau dans les CEE est un résultat inattendu - les CEE ne sont pas des neurones (bien que partageant des caractères communs) - qui vient alimenter le rôle crucial de l'axe intestin-cerveau dans les maladies neurodégénératives.

V. CONCLUSION

Mon parcours depuis l'obtention du doctorat montre des changements thématiques forts. La physiologie est néanmoins restée au cœur de mes approches scientifiques et je continue à être animée par cette discipline. Depuis mon arrivée à l'UMR PhAN, j'ai contribué à l'apport de connaissances sur la programmation de l'axe intestin-cerveau par l'environnement précoce (dénutrition protéique maternelle, supplémentation néonatale en oligosides prébiotiques, hyperglycémie *in utero*, obésité maternelle) et ses effets sur le devenir métabolique et le comportement alimentaire de la descendance. Dans l'axe intestin-cerveau, les cellules entéro-endocrines, sentinelles de la lumière intestinale et le nerf vague, relais majeur de la communication bi-directionnelle entre ces deux organes ont été mes cibles d'études. Ces travaux ont donné lieu à plusieurs co-encadrements de thèses et de M2 associés aux publications. Je n'ai pour autant pas terminé de répondre à ma question centrale : les CEE sont-elles programmables et quel est impact de cette programmation à long-terme sur des fonctions clefs de ces cellules (prise alimentaire, homéostasie glucidique) ? La mise en place d'un rat transgénique pCCK-GFP, outil de choix pour l'étude de cette lignée de CEE, devait m'apporter des réponses mécanistiques à ces questions. Les difficultés méthodologiques rencontrées (tri cellulaire, microdissection laser sur tissu frais, organoïdes) ont considérablement ralenti l'avancement de mes travaux mais je ne renonce pas à trouver des solutions pour y donner suite. Les résultats originaux obtenus sur l'expression de Tau dans les CEE ouvrent la voie vers des recherches nouvelles sur ces cellules et pour ma part m'amènent à questionner le rôle de l'environnement périnatal sur l'expression de cette protéine au cours de la vie et du vieillissement. Les années à venir seront donc consacrées à ces questions et l'habilitation à diriger des recherches me permettra de poursuivre avec plus d'autonomie et de gagner une visibilité qui me fait défaut actuellement. La formation des jeunes à et par la recherche a toujours été une mission essentielle comme en témoignent mes co-encadrements passés et en cours ainsi que les formations à l'encadrement doctoral auxquelles j'ai participé. Mon implication dans l'enseignement supérieur n'a jamais cessé depuis ma première année de thèse. J'aurai certainement dû m'engager plus tôt dans l'obtention de ce diplôme, non seulement pour moi mais aussi pour l'UMR PhAN que j'ai le plaisir de diriger aujourd'hui en tant qu'adjointe.

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VI. LISTE DES PUBLICATIONS ET PRODUITS

1. ARTICLES SCIENTIFIQUES

1.1 Articles publiés (revues avec comité de lecture)

2023

1. Chapelet, G., Béguin, N., Castellano, B., Grit, I., de Coppet, P., Oullier, T., Neunlist, M., Blottière, H., Rolli-Derkinderen, M., Le Dréan, G. (co-dernier auteur), Derkinderen, P. (2023). Tau expression and phosphorylation in enteroendocrine cells. *Frontiers in Neuroscience*. hPMC10272410. https://nantes-universite.hal.science/hal-04163668

2022

2. Tavares, G.A., Torres, A., Le Dréan, G., **Queignec, M.**, Castellano, B., Tesson, L., Remy, S., Anegon, I., Pitard, B., Kaeffer, B. (2022). Oral Delivery of miR-320-3p with Lipidic Aminoglycoside Derivatives at Mid-Lactation Alters miR-320-3p Endogenous Levels in the Gut and Brain of Adult Rats According to Early or Regular Weaning. *International Journal of Molecular Science*. 24(1):191. doi: 10.3390/ijms24010191. PMID: 36613633; PMCID: PMC9820440. https://hal.inrae.fr/inserm-03925111

2021

3. **Pocheron, A.-L.**, Le Dréan, G., (co-premier auteur), Billard, H., Moyon, T., Pagniez, A., Heberden, C., Le Chatelier, E., Darmaun, D., Michel, C., Parnet, P. (2021). Maternal Microbiota Transfer Programs Offspring Eating Behavior. *Frontiers in Microbiology*. 12:672224. doi: 10.3389/fmicb.2021.672224. PMID: 34211445; PMCID: PMC8239415. https://hal.inrae.fr/hal-03271317

2019

4. Le Dréan, G., (Auteur de correspondance), **Pocheron, A.L.**, Billard, H., Grit, I., Pagniez, A., Parnet, P., Chappuis, E., Rolli-Derkinderen, M., Michel, C. (2019). Neonatal consumption of oligosaccharides greatly affects L cell density without significance consequence for adult eating behavior. *Nutrients. 11(9):*1967. doi: 10.3390/nu11091967. https://hal.inrae.fr/hal-02627809

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5. Aubert, P., Oleynikova, E., Rizvi, H., **Ndjim, M**., Le Berre-Scoul, C., Grohard, P. A., Chevalier, J., Segain, J.-P., Le Drean, G., Neunlist, M., Boudin, H. (Auteur de correspondance) (2018). Maternal protein restriction induces gastrointestinal dysfunction and enteric nervous system remodeling in rat offspring. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, fj201800079R. , DOI : 10.1096/fj.201800079R https://prodinra.inra.fr/record/440566

6. Desir-Vigne, A., **Haure-Mirande, J.-V.**, De Coppet, P., Darmaun, D., Le Drean, G., Segain, J.-P. (Auteur de correspondance) (2018). Perinatal supplementation of 4-

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2017

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1.2 Articles publiés (revue sans comité de lecture)

2018

32. Parnet, P., Michel, C., Pocheron, A.-L., Amarger, V., Le Dréan, G. (2018). Les microbiotes transférés de la mère jouent-ils un rôle dans l'origine développementale de la santé et des maladies ? *Innovations Agronomiques, 65*, 1-14.

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33. Le Dréan G. (2010). Flores fromagères. La PCR quantifie les mycéliums. *Process alimentaire*, 1270, 93.

2. OUVRAGES, CHAPITRES D'OUVRAGES, RAPPORTS DIPLÔMANTS

1. Le Dréan, G. et Derkinderen, M. « Enseignement et Formation, Troisième cycle » in : L'Institut des maladies de l'Appareil Digestif du CHU de Nantes. Eds du Grand Métier, 68 p.

3. COMMUNICATIONS À DES CONGRÈS ET COLLOQUES

3.1 Communications invitées

1. [Présentation orale] Le Dréan G. Nutrition périnatale et troubles du comportement alimentaire. Assises Jeunes Chercheurs du département AlimH de l'INRA, 14-15 octobre 2015, Cannes
2. [Présentation orale] Le Dréan G. Microbiotes maternels et comportement alimentaire de la descendance. Congrès annuel Mibiogate. Etudes des barrières d'organes et leur microbiote, 10-11 mars 2022, Nantes.
3. [Présentation orale] Le Dréan G. Transmission maternelle du risque métabolique à la descendance. Journées Francophones de Nutrition 6-8 décembre 2023, Marseille.

3.2 Communications (liste réduite à la période INRAE. 2009-2023)

Gras : étudiant encadré ou co-encadré

Souligné : orateur

2023

1. [Présentation orale] **Bobin P.**, David-Sochard A., Castellano,B., Grit I., Gandon A., Croyal M., Gourdel M., Le Dréan G., Alexandre-Gouabau M-C.. L'homéostasie énergétique de la descendance exposée *in utero* à une hyperglycémie maternelle est modulée à long terme selon la composition du lait maternel reçu pendant la période d'allaitement. SF-DOHaD 16-17 novembre 2023, Rennes
2. [Poster] Le Dréan G., **Bobin P.**, Legrand M., Castellano B., Gandon A., Alexandre-Gouabau M-C. Exploration comportementale des troubles de type TSA et TDAH dans un modèle murin de diabète gestationnel : effet du régime maternel pendant la lactation. SF-DOHaD 16-17 novembre 2023, Rennes
3. [Présentation orale] **Bobin P.**, Castellano B., Lefebvre A., Grit I., David-Sochard,A., Gourdel M., Croyal M., Le Dréan G., Alexandre-Gouabau M-C. L'exposition in utero à l'hyperglycémie maternelle impacte le métabolome/lipidome plasmatique de la descendance à court terme et son homéostasie énergétique à long terme selon la composition du lait maternel reçu pendant la période d'allaitement. Journées Francophones de Nutrition 6-8 décembre 2023, Marseille.

2022

4. [Présentation orale] Le Dréan G., de Coppet P., Fromentin S., **Houssais B., Pocheron**, **A-L.** Canlet C., Moyon T., Plaza-Oñate F., Heberden C., Le Chatelier E., Darmaun D., Parnet P., Michel C. Impact d'un transfert de micobiotes maternels et de leurs métabolites sur l'expression de marqueurs entéro-endocrines chez le raton. 39ème réunion du CECED, 24-25 mars 2022, Lyon
5. [Poster numérique commenté] **Bobin P.**, de Coppet P., David-Sochard A., Grit I., Croyal M., Abderrahmani A., Robitaille J., Le Dréan G., Alexandre-Gouabau M-C. Le diabète gestationnel induit des modifications de la composition du lait maternel qui pourraient avoir des répercussions sur l'homéostasie énergétique de la descendance. Journées Francophones de Nutrition, 16-18 novembre 2022, Toulouse
6. [Poster] Parnet P, Le Dréan G., Fromentin S., **Pocheron A-L.**, Billard B., Moyon T., Amarger V., Heberden C., Le Chatelier E., Michel C. NEONATAL TRANSFER OF MATERNAL MICROBIOTA HAS A LASTING EFFECT ON THE FEEDING BEHAVIOR OF THE

OFFSPRING. DOHaD World Congress 2022, International society for Developmental Origins of Health and Disease, Aug 2022, Vancouver, Canada https://hal.inrae.fr/hal-03973492

7. [Poster] Le Dréan G., de Coppet P., Fromentin S., **Pocheron A-L.**, Canlet C., Moyon T., Heberden C., Le Chatelier E., Darmaun D., Parnet P., Michel C. MATERNAL MICROBIOTAS TRANSFER IMPACTS MICROBIOTA-GUT-BRAIN AXIS IN OFFSPRING. *DOHaD World Congress 2022*, International society for Developmental Origins of Health and Disease, Aug 2022, Vancouver, Canada. 2p. ⟨hal-03973514⟩
8. [Poster] Alexandre-Gouabau MC, Le Dréan G, Kaeffer B, Abderrahmani A, De Coppet P, Bobin P, Croyal M, De Luca A, Hanckard R, Robitaille J. Gestational Diabetes Mellitus modifies human breast milk content in insulin sensitivity-regulators. *DOHaD World Congress 2022*, International society for Developmental Origins of Health and Disease, Aug 2022, Vancouver, Canada.

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9. [Poster] **Pocheron A-L.**, Le Dréan G., Michel C., Parnet P. Etude du rôle des microbiotes maternels dans la transmission du risque d'obésité à la descendance. Congrès annuel scientifique Mibiogate, 26 mars 2021, Nantes

2020

10. [Présentation orale] Tavares G.A., Torres A., **Queinec M.**, Pitard B., Le Dréan G., Grit I., Gandon A., Castellano B., de Souza S.L., Kaeffer B. Effects of Early Weaning and Postnatal Oral Supplementation of Mir-320-3p On MRNA And MiRNA Expression in Different Brain Areas in Juvenile Rats of Both Sexes. Latin America DOHAD on-line congress, October 13-16, 2020 (Abstract n°3433; Honor Award attributed to Gabriel)
11. [Poster] Torres A., Tavares G.A., **Queignec M.,** Pitard B., Le Dréan G., Grit I., Gandon A., Castellano B., de Souza S.L., Kaeffer B. Nutritional Epigenetic Re-Programming Brain Expression Profiling on Rat Pups after the Supplementation of miR-375-3p Embedded in biomimetic vehicles. Latin America DOHAD on-line congress, October 13-16 2020, Rio de Janeiro, Brazil

2019

12. [Présentation orale] **Pocheron A-L.,** Le Dréan G., Le Chatelier E., Michel C., Parnet P. (2019-06-21). *Maternal obesogenic microbiota modified early fecal microbiota and feeding behavior of transplanted rat offspring?* Presented at : Mibiogate "Physiologie et Pathologie des barrières : interaction avec les microbiotes", Nantes, France (2019-06-21 - 2019-06-21), https://hal.inrae.fr/hal-02789018
13. [Présentation orale] **Pocheron A-L.,** Le Dréan G., Le Chatelier E., Michel C., Parnet P. (2019-09-12). *Neonatal intestinal microbiota composition impacts rat feeding behavior*. Presented at : Congrès international Recherche Fédération Française Anorexie Boulimie FFAB-R, Paris, France (2019-09-12 - 2019-09-12), https://hal.inrae.fr/hal-02737518
14. [Poster] Michel C., **Pocheron A-L**., Billard H., Le Dréan G., Parnet P. (2019-10-31). Changes in early intestinal microbiota induced by transfers of vaginal, milk-associated and fecal microbiota from pregnant mothers do not program intestinal microbiota in adult rats. Presented at: International Conference World of Microbiome: Pregnancy, Birth and Infancy (WoMPBI), Milan, Italie (2019-10-31 - 2019-11-02), https://hal.inrae.fr/hal-02736187

2018

15. [Poster] **Pocheron A-L**., Le Dréan, G., Michel, C. Parnet, P. (2018). *Etude du rôle des microbiotes maternels dans la transmission du risque d'obésité à la descendance***.** Presented at Colloque Mibiogate "Physiologie et Pathologie des barrières : interaction avec les microbiotes", Journées scientifiques de l'Université de Nantes, FRA (2018-06-01)
16. [Présentation orale] **Pocheron A-L**., Le Dréan G., Ben Necib, R., Michel, C. Parnet, P. Le transfert néonatal de microbiotes associés ou non à l'obésité modifie le comportement alimentaire de la descendance à court et moyen termes chez le rat. 4èmes Journées de la SF-DOHaD, 8-9 novembre 2018, Grenoble.

2017

17. [Présentation orale] **Pocheron, A.-L.,** Le Dréan, G., Parnet, P., Michel, C. (2017). Does early intestinal microbiota modification by oligosaccharides program adult eating behavior in rats? Presented at Journées nationales sur les troubles du comportement alimentaire, Rouen, FRA (2017-05-18 - 2017-05-19). http://prodinra.inra.fr/record/402575
18. [Présentation orale] **Ndjim M.,** Aubert, P., De Coppet, P., Bonnet C., Poupeau G., Neunlist M., Le Dréan G. (2017). Maternal protein restriction in young rats alters long chain fatty acid sensing by duodenal entero-endocrine cells, leading to increased-intestinal permeability. Presented at 9. European Symposium on Neurogastroenterology and Motility, Cork, IRL (2017-08-24 - 2017-08-26). http://prodinra.inra.fr/record/400901
19. [Poster] **Ndjim M.,** Mériaux R., Falcon L., Segain J.-P., Remy S., Tesson L., Le Dréan G. (2017). Maternal protein restriction increases duodenal entroendocrine cells in young rats. Presented at 9. World congress developmental origins of health and disease (DOHAD), Rotterdam, NLD (2017-10-15 - 2017-10-18). http://prodinra.inra.fr/record/400900
20. [Présentation poster] **Ndjim, M.**, Aubert, P., De Coppet, P., Bonnet, C., Poupeau, G., Neunlist, M., Le Dréan, G. (2017). *Intrauterine growth retardation in rats alters palmitoleate sensing by duodenal entero-endocrine cells, leading to increased-intestinal permeability*. Presented at 9. World congress developmental origins of health and disease (DOHAD), Rotterdam, NLD (2017-10-15 - 2017-10-18). http://prodinra.inra.fr/record/402155

2016

21. [Poster] Desir-Vigne, A., **Haure-Mirande, V**., De Coppet, P., Le Dréan, G., Bonnet, C., Segain, J.-P. (2016). Rôle du stress du réticulum endoplasmique dans les modifications épigénétiques de l'épithélium colique induit par un retard de croissance intra-utérin chez le rat. *Nutrition Clinique et Métabolisme, 30* (1). Presented at Les Journées Francophones de Nutrition Nutrition Clinique et Métabolisme, Marseille, FRA (2015-12-09 - 2015-12-11)., DOI : 10.1016/j.nupar.2016.01.008 http://prodinra.inra.fr/record/355882
22. [Présentation orale] **Ndjim, M.,** De Coppet, P., Poupeau, G., Le Dréan, G. (2016). *Duodenal fatty acid sensing is not altered by perinatal malnutrition in adult rat born with intrauterine growth retardation*. Presented at 3ème congrès SF DOHaD 2016, Paris, FRA (2016-12-01 - 2016-12-02). http://prodinra.inra.fr/record/402166
23. [Présentation orale] Oleynikova, E., Aubert, P., Rizvi, H., Le Berre-Scoul, C., Grohard, P. A., Chevalier, J., Segain, J.-P., Le Dréan, G., Neunlist, M., Boudin, H. (2016). Intestinal dysfunctions induced by intrauterine growth retardation are associated with altered autophagy in the enteric nervous system. In: Special Issue: 2nd Federation of Neurogastroenterology and Motility Meeting (p. 87-87). *Neurogastroenterology and Motility, 28* (Issue Supplement S1). Presented at 2. Federation of Neurogastroenterology and Motility Meeting, San Francisco, California, USA (2016-08-25 - 2016-08-28). Hoboken, USA : Wiley-Blackwell. 116 p., DOI :

10.1111/nmo.12881. http://prodinra.inra.fr/record/386276

24. [Poster] Le Dréan, G., **Olier, A.,** Rolli Derkinderen, M., Michel, C. (2016). Oligosaccharides consumption affects neonatal gut connectome. In: Proceedings from the 8th Probiotics, Prebiotics & New Foods for Microbiota and Human Health meeting held (p. 231-232). *Journal of Clinical Gastroenterology, 50* (Supplement 2 November/December 2016). Presented at 8. Probiotics, Prebiotics & New Foods for Microbiota and Human Health meeting, Rome, ITA (2015-09-13 - 2015-09-15). Philadelphie, USA : Lippincott Williams & Wilkins. 233 p., DOI : 10.1097/MCG.0000000000000691. http://prodinra.inra.fr/record/386247
25. [Poster] **Ndjim, M.,** Aubert, P., De Coppet, P., Grit, I., Bonnet, C., Poupeau, G., Neunlist, M., Le Dréan, G. (2016). *Intrauterine growth retardation (IUGR) promotes sensitivity to the satietogenic effect of palmitoleate in young rats*. Presented at RegPep 2016, Rouen, FRA (2016-07-11 - 2016-07-15). http://prodinra.inra.fr/record/402131
26. [Présentation orale] **Ndjim M.,** de Coppet P., Poupeau G., Le Drean G. Duodenal fatty acid sensing is not altered by perinatal malnutrition in adult rat born with intrauterine growth retardation. Colloque de la SF-DOHaD, Paris, 1-2 décembre 2016.
27. [Présentation orale] Michel C., **Pocheron A. L.,** Reufflet A., **Gouyon E.**, Morel F., Le Dréan G. Early oligosaccharide consumption affects enteroendocrine cells maturation but fails to program adult eating behavior. Colloque de la SF-DOHaD, Paris, 1-2 décembre 2016.

2015

28. [Présentation orale] Desir-Vigne A., De Coppet P., Bonnet C., Le Dréan G., Segain, J.-P. (2015). *Rôle du stress du réticulum endoplasmique dans les modifications épigénétiques associées aux altérations de la barrière épithéliale colique*. Presented at 34. Réunion annuelle du Club d'Etudes des Cellules Epithéliales Digestives (CECED), Nantes, FRA (2015-03-26 - 2015-03-27). http://prodinra.inra.fr/record/402572
29. [Poster] Michel C., **Olier A.**, Poupeau G., Castellano B., Rolli Derkinderen M., Le Dréan G. (2015). Does early oligosaccharides consumption affect gut-brain axis maturation? Presented at 15. IFAC Symposium on System Identification, Budapest, HUN (2015-06-23 - 2015-06-25). http://prodinra.inra.fr/record/402170
30. [Présentation orale] Désir-Vigné A., **Haure-Mirande V.**, de Coppet P., Bonnet C., Le Drean G., Segain J.P. Role of the endoplasmic reticulum stress in the loss of histone acetylation in colonic epithelium induced by intrauterine growth retardation. Presented at Epinantes, Nantes, 6-7 octobre 2015.
31. [Présentation orale] Désir-Vigne A., de Coppet P., Bonnet C., Le Dréan G., Segain J-P. Rôle du stress du réticulum endoplasmique dans les modifications épigénétiques associées aux altérations de la barrière épithéliale colique. (CECED), Nantes, 26-27 mars 2015.
32. [Poster] Le Dréan G., **Olier A.**, Rolli-Derkinderen M., Michel C. Oligosaccharide consumption affects neonatal gut connectome. Presented at 8th Probiotics, Prebiotics & New Foods - for microbiota and human health" Rome, September 13-15, 2015
33. [Poster] Michel C., **Olier A.**, Poupeau G., Castellano B., Rolli-Derkinderen M., Le Dréan G. Does early oligosaccharides consumption affect gut brain axis maturation? ISC Probiotics and Prebiotics, 23th-25th June 2015

2014

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(volume horaire annuel)

1. [Cours] Université de Rennes 1, L2 & L3 Biologie cellulaire et physiologie, Physiologie Animale (TP & TD 200 h)
2. [Cours] Université de Brest, Cycle ingénieur ESIAB, 2ème année, Qualité des productions animales (84 h)
3. [Cours] Université de Brest, Cycle ingénieur ESIAB, 3ème année, Nutrition Humaine (26 h)
4. [Cours] Université de Brest, Cycle ingénieur ESIAB, 1ère année, Biochimie analytique et Biologie moléculaire (TP, 100h)
5. [Cours] Université de Nantes, Master2, Nutrition et Sécurité Alimentaire « L'axe intestin cerveau dans la régulation de la prise alimentaire » (1h20)
6. [Cours] Université de Nantes, Master2, Biologie Santé, parcours Biologie, Biotechnologie, Recherche Thérapeutique, option Physiopathologies de l'axe intestin cerveau : « Cellules entéro-endocrines, nerf vague et obésité » (1h20)

7. [Cours] Université de Nantes, Master 1, Biologie Santé, UE Physiologie Digestive et nutritionnelle « Fonction endocrine intestinale » (1h20). UE Sysdige M1 MICAS depuis 2023
8. [Cours] Nantes Université, Cursus Master Doctorat (Graduate Program) MICAS, UE Neuropsy « L'axe intestin-cerveau dans la régulation de la prise alimentaire : rôle du nerf vague »
9. [Cours] Nantes Université, Cursus Master Doctorat (Graduate Program) MICAS, UE Méthodologie « Analyse des comportements alimentaires » atelier animé avec P. Parnet.

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VII. ANNEXES

Annexe 1. Publication #9.

The FASEB Journal article fj.13-234203. Published online November 15, 2013.

The FASEB Journal • Research Communication

Visceral adipose tissue and leptin increase colonic epithelial tight junction permeability *via* a RhoA-ROCK-dependent pathway

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ABSTRACT **Proinflammatory cytokines produced by immune cells play a central role in the increased intestinal epithelial permeability during inflammation. Expansion of visceral adipose tissue (VAT) is currently considered a consequence of intestinal inflammation. Whether VAT *per se* plays a role in early modifications of intestinal barrier remains unknown. The aim of this study was to demonstrate the direct role of adipocytes in regulating paracellular permeability of colonic epithelial cells (CECs). We show in adult rats born with intrauterine growth retardation, a model of VAT hypertrophy, and in rats with VAT graft on the colon, that colonic permeability was increased without any inflammation. This effect was associated with altered expression of tight junction (TJ) proteins occludin and ZO-1. In coculture experiments, adipocytes decreased transepithelial resistance (TER) of Caco-2 CECs and induced a disorganization of ZO-1 on TJs. Intraperitoneal administration of leptin to lean rats increased colonic epithelial permeability and altered ZO-1 expression and organization. Treatment of HT29-19A CECs with leptin, but not adiponectin, dose-dependently decreased TER and altered TJ and F-actin cytoskeleton organization through a RhoA-ROCK-dependent pathway. Our data show that adipocytes and leptin directly alter TJ function in CECs and suggest that VAT could impair colonic epithelial barrier.—Le Dréan, G., Haure-Mirande, V., Ferrier, L., Bonnet, C., Hulin, P., de Coppet, P., Segain, J.-P. Visceral adipose tissue and leptin increase colonic epithelial tight junction permeability *via* a RhoA-ROCK-dependent pathway. *FASEB J.* 28, 000–000 (2014). www.fasebj.org**

Key Words: adipocytesactin cytoskeleton · barrier · adipokines

THE MONOLAYER OF POLARIZED epithelial cells lining the intestine forms a crucial barrier that limits host contact with the massive luminal load of dietary antigens and microbes. Intestinal epithelial barrier function relies on the integrity of intercellular protein junctions, including adherent and tight junctions (TJs), which maintain epithelial cohesion, polarity, and paracellular sealing (1). TJs are the most apical structures of the lateral plasma membrane of epithelial cells and are primarily involved in the regulation of paracellular permeability (1, 2). TJs are composed of transmembrane proteins, such as claudins and occludin, which interact with many different cytoplasmic proteins, such as ZO-1 (1). ZO-1, in turn, links TJ proteins to F-actin filaments of the apical perijunctional actomyosin ring (3). Mounting evidence indicates that organization and contraction of the perijunctional F-actin cytoskeleton, which are mainly regulated by the myosin light-chain kinase (MLCK) and the RhoA/ Rho-kinase (ROCK) pathway, play an important role in controlling TJ structure and function (4–8).

Increased permeability of the intestinal epithelial barrier is frequently found in inflammatory bowel disease (IBD; ref. 4) but has also been observed in both genetic and dietary models of obesity, along with gut inflammation (9–12). Increased intestinal permeability allows the entry of luminal antigens and bacterial products (*e.g.*, lipopolysaccharide, LPS) that can perpetuate inflammatory responses in IBD and obesity (2, 10). It is believed that decreasing intestinal permeability may be a therapeutic strategy to treat or prevent inflammatory responses (2, 4). Thus, identifying cellular and molecular mechanisms affecting integrity and function of the intestinal epithelial barrier represents an important challenge.

Abbreviations: CEC, colonic epithelial cell; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IL, interleukin; IUGR, intrauterine growth restriction; LBP, LPS-binding protein; LPS, lipopolysaccharide; ROCK, Rho-kinase; TER, transepithelial resistance; TNF, tumor necrosis factor; TJ, tight junction; VAT, visceral adipose tissue

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During inflammation, immune and intestinal epithelial cells (IECs) are the main effectors secreting proinflammatory cytokines, such as tumor necrosis factor (TNF)-α or interleukin (IL)-1β, known to increase paracellular permeability of IECs (4). However, several studies have also identified the visceral adipose tissue (VAT) as an important source of cytokines in IBD, especially in Crohn's disease (13, 14). It has been suggested that expansion of an inflamed VAT is a consequence of inflammation and enhanced permeability of intestinal barrier (9, 12). This VAT is characterized by infiltrating macrophages that may amplify or perpetuate inflammation by producing proinflammatory cytokines (13–15). VAT also secretes numerous proteins (adipokines) with a broad spectrum of activities, including the regulation of metabolism, appetite, or energy balance, but also immune and inflammatory responses (16, 17). Alterations of adipokine levels (*e.g.*, leptin, adiponectin) are found in VAT and serum of patients with IBD and in experimental colitis (14, 18, 19).

The role of VAT and adipokines on intestinal epithelial barrier function remain poorly characterized. Yet, it has been shown that the colonic epithelium expresses adiponectin and leptin receptors (20–22) and that leptin stimulates mucin secretion and proliferation of colonic epithelial cells (CECs), *in vitro* and *in vivo* (22, 23). The effect of leptin on paracellular permeability of CECs is presently unknown. Recently, a positive association between intestinal (proximal and distal) permeability and visceral adiposity was found in lean and overweight healthy women, in the absence of gastrointestinal disorders (24). The question of a potential implication of VAT in the increased intestinal permeability, therefore, is relevant.

The present study was thus undertaken to determine whether VAT and adipokines (leptin and adiponectin) could act directly on CECs to alter TJ and paracellular permeability. We used a model of adult rats born with a low birth weight due to intrauterine growth restriction (IUGR), which have a predisposition to develop excess VAT in adulthood even when fed a standard chow (25, 26). The role of VAT on colonic permeability was also investigated *in vivo* by grafting exogenous VAT on the colon of lean rats. Intraperitoneal administration of leptin was performed to study its effect on colonic permeability *in vivo*. The direct effect of adipocytes and leptin on paracellular permeability and TJ function was studied *in vitro* using monolayers of human CECs (HT29 and Caco-2). Our results showed that VAT and leptin increase epithelial colonic permeability by altering TJs.

MATERIALS AND METHODS

Animals and experiments

All experiments were conducted in accordance with the European Union regulations for the care and use of animals for experimental procedures (2010/63/EU). The protocol was approved by the Committee on the Ethics in Animal Experiments of Pays de la Loire, France (project number 2012.174). Adult female and male Sprague-Dawley rats and male Zucker rats (*ZUC-Lepr*^{fa}; Charles River, Lyon, France) were housed individually under standard laboratory conditions.

IUGR

IUGR was generated in Sprague-Dawley female rats, as described previously (27, 28). Briefly, 6 pregnant dams were fed either a diet containing 20% protein (control group) or an isocaloric diet containing 8% protein (IUGR group) throughout pregnancy and lactation. Control (n=10) and IUGR (n=10) rat offspring were weaned at 21 d of age and received standard laboratory chow (AIN93; Safe, Augy, France). At 240 d of age, rats were euthanized by CO_2 asphyxiation, followed by cervical dislocation. After laparotomy, the whole colon was removed, and the cecum and proximal colon were collected for permeability measurement, protein extraction, and RNA isolation, as described below. Fat pads, including pericolonic, retroperitoneal, and mesenteric, were collected, weighed, and stored at −80°C. Blood was collected from the heart, and serum was prepared for ELISA measurement of leptin, adiponectin, and LPS binding protein (LBP).

VAT transplantation

Three 11-wk-old donor Sprague-Dawley male rats were fed a high-fat diet (Purified diet 230HF; Safe) for 2 wk to induce a mild visceral adiposity, while six 11-wk-old lean recipient rats received a standard chow. The day of surgery, rats were anesthetized (isoflurane 2%), and VAT was collected (800 mg) from the ceco-colic junction of donor rats and transplanted near the ceco-colic junction of 3 recipient rats under aseptic conditions. The 3 other rats were used for the sham surgery that consisted of suturing the ceco-colic junction without transplant. Grafted and sham-treated rats were then fed a standard chow diet. At 3 d following surgery, rats were euthanized, and cecum and proximal colon were collected for permeability measurement in Ussing chambers, histology, immunohistology, protein extraction and RNA. Portal blood was collected, and serum was prepared for ELISA measurement of LBP levels. VAT transplantation experiment was repeated twice, and data were pooled to obtain n = 6.

Intraperitoneal injection of leptin

Two groups of six 3-wk-old Sprague-Dawley male rats received either an intraperitoneal injection (100 μl) of 50 μg recombinant rat leptin (Preprotech, Neuilly sur Seine, France), or vehicle (0.9% NaCl). At 6 h after the injection, rats were euthanized, and cecum was collected and mounted in Ussing chambers for measurement of colonic permeability. Proximal colon tissue samples were also collected for immunohistology and RNA isolation. Intestinal permeability was also assessed *in vivo* as described before (29). Briefly, 2 groups of 10 Sprague-Dawley rats and 2 groups of 10 *fa/fa* Zucker rats received i.p. injections of leptin or saline. Immediately afterward, all animals received by gavage 0.7 μCi ^{51}Cr-EDTA in 0.5 ml 0.9% NaCl, and urine was collected for 6 h. Then, radioactivity was measured in urine with a γ counter, and intestinal permeability was expressed as the percentage of total radioactivity administered recovered in urine.

Ussing chamber experiments

Colonic permeability was evaluated in a Ussing chamber, as described previously (29). Briefly, cecum tissue samples (exposed area, 0.5 cm^2) were mounted (in duplicate) in Ussing chambers containing Krebs buffer gassed with 95% O_2/5% CO_2 and connected to a voltage-clamp apparatus (Physiological Instruments, San Diego, CA, USA). The transepithelial resistance (TER; Ω/cm^2) was recorded throughout the experiment. After 20 min, FITC-labeled dextran-4-kDa (FD4; Sigma, Saint-Quentin Fallavier, France) was added at the mucosal side (2 mg/ml), and fluorescence was measured at the serosal side after 60 min. Results were expressed as the flux of dextran crossing the epithelial barrier (μg/h).

ELISA

Serum was assayed for total leptin and adiponectin using specific ELISA kits from Millipore SAS (Molsheim, France) and AbCys SA (Eurobio, Courtaboeuf, France), respectively. LPS-binding protein was quantified using an ELISA kit (Hycult Biotech, Uden, The Netherlands). Interleukin (IL)-6 and TNF-α concentrations were measured in protein lysates of proximal colon and of pericolonic adipose tissue using ELISA kits (AbCys, Eurobio, Courtaboeuf, France). Protein lysates were prepared by grinding the tissues in PBS containing 0.5% Triton X-100 and a protease inhibitor cocktail (Sigma) using a tissue homogenizer (Precellys, Bertin Technologies, Aix-en-Provence, France).

Western blot analysis

Protein lysates prepared from proximal colon, as described above, were analyzed by Western blot using an anti-ZO-1 rabbit polyclonal antibody (1:500; Zymed, now Invitrogen, Carlsbad, CA, USA) and an anti-β-actin mouse monoclonal antibody (1:10,000; Sigma). Immunoreactive bands were visualized with DyLight 680 and 800-conjugated antibodies (KPL, Gaithersburg, MD, USA), respectively. Band intensities were quantified by infrared scanning densitometry (Odyssey Imaging Systems; Li-Cor, Lincoln, NE, USA).

Cell culture and coculture experiments

Human CECs HT29-19A (30) and Caco2 cells (American Type Culture Collection, LGC Standards, Molsheim, France) were cultured in DMEM (Invitrogen, Life Technologies, Saint-Aubin, France) supplemented with 10% FCS, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 2 mM glutamine at 37°C in a 5% CO_2 atmosphere. To obtain monolayers of fully differentiated and polarized epithelial cells, CECs were seeded in cell culture inserts (Transwell filters, 0.4-μm porosity; Costar; Corning, Tewksbury, MA, USA) at a density of 2×10^5 cells/cm^2 in 12-well plates. Cell cultures were maintained at confluence for 8–10 d with culture medium refreshed every day in both basal and apical compartments. At this time, average TER values of individual cell monolayers were 450 ± 60 and 1100 ± 100 Ω/cm^2, for HT29-19A and Caco2, respectively. The day of the experiment, basal medium was replaced with medium containing 10 ng/ml of the following adipokines: leptin, adiponectin, IL-6, TNF-α (Peprotech, Tebu-bio, Le Perray-en-Yveline, France) at indicated time and doses. In separate experiments, cells were pretreated with the ROCK inhibitor, Y-27632 (10 μM) before the addition of leptin (10 ng/ml). Cell monolayers were also used in coculture experiments with PAZ-6 adipocytes.

The human preadipocyte cell line PAZ-6 (31) was cultured in DMEM/Ham culture medium: DMEM/Ham's F12 (1:1, vol/vol) supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a 5% CO_2 atmosphere. For differentiation, PAZ-6 cells were cultured in DMEM/Ham's F12 supplemented with 15 mM HEPES, 33 μM biotin, 17 μM pantothenate, 1 nM tri-iodothyronine, 100 nM dexamethasone, 1 μM pioglitazone, 850 nM insulin, and 0.25 mM 3-isobutyl-1 methyl-xanthine (all products from Sigma), in 12-well plates for 15 d. The medium was changed every other day. Adipocyte differentiation was monitored by coloration of intracellular lipid droplets using Oil Red O and by analysis of leptin and adiponectin mRNA expression by RT-quantitative PCR (RT-qPCR). The day before coculture, the medium of the basal compartment of CEC inserts and the medium of differentiated adipocyte cultures were replaced by DMEM/Ham medium. The next day, Transwell filters containing monolayers of CECs were placed in 12-well plates containing either DMEM/Ham medium alone, preadipocytes, or differentiated adipocytes (PAZ-6), and cocultures were maintained at 37°C for 48 h, and TER was measured at indicated times.

Measurement of paracellular permeability of CECs

TER of CEC monolayers cultured on Transwell filters was measured with a volt-ohm meter (EVOM, Millicell; Millipore, Molsheim, France). The change in TER was expressed as ΔTER from the initial TER value measured at time 0. Paracellular permeability was also measured by adding FITC-dextran (2 mg/ml) in the apical medium. After 1 h, fluorescence was measured in the basal medium using a fluorometer (485-nm excitation and 516-nm emission wavelengths).

Real-time qPCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Life Technologies) and treated for 45 min at 37°C with RQ1 DNase (Promega, Charbonnières-Les-Bains, France). RNA (1 μg) was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen). The cDNA solution (1 μl) was subjected to real-time qPCR in a Bio-Rad iCycler iQ system using the qPCR SYBR Green MasterMix (Fermentas, Courtaboeuf, France). Quantitative PCR consisted of 45 cycles, 30 s at 95°C and 30 s at 60°C each. Primer sequences are presented in **Table 1**.

Immunofluorescence staining

Immunofluorescence staining of colonic tissues and cell cultures was performed as described previously (32, 33). Briefly, rat colonic tissue sections were microwave-heated in citrate buffer for antigen retrieval and then incubated with anti-occludin or anti-claudin-1 rabbit polyclonal antibodies (1:100; Zymed, Life Technologies) overnight at 4°C. After washes, sections were then stained with goat anti-rabbit biotinylated antibody (Vector Laboratories) followed by Alexa Fluor 488-conjugated streptavidin antibody (Molecular Probes, Life Technologies). Nuclei were counterstained with Hoechst. For cyto-immunofluorescence, CEC monolayers were fixed at the end of experiments with either PBS containing 2% paraformaldehyde at room temperature, or with methanol at −20°C, for F-actin or ZO-1 staining, respectively. F-actin cytoskeleton was stained with Alexa Fluor 488-phalloidin (Molecular Probes, Life Technologies). ZO-1 was stained with a rabbit anti-ZO-1 antibody (Zymed) overnight at 4°C, followed by incubation with CY3-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories). Tissue sections and cells were mounted in

TABLE 1. *Sequences of primers used for RT-qPCR analysis*

Gene	Forward primer	Reverse primer
Human		
Adiponectin	CTTCCATCGCCAAGTTCGTCTG	CATCGTGGTTGCCGGTGTTG
Adiponectin R	ACGGTGGAACTGGCTGAACTG	CCGCACCTCCTCCTCTTCTTC
IL-6	TTGGACACTCACACGGACACTC	CGGCTGGAGGAGGCTTTGG
IL-6 R	TTGGACACTCACACGGACACTC	CGGCTGGAGGAGGCTTTGG
Leptin	TGTGCGGATTCTTGTGGCTTTG	GGAGGAGACTGACTGCGTGTG
Leptin R	TGAAGCCACTGCCTCCATCC	CGCACCTGAACAGCATAGACTG
TNF-α	CACCATCCGCACCTCCATCC	CCCGTCACAAGCCCCGTAG
TNF-α R	CTAGCAGCCGCCTACTTGGTG	CGTCCCTCATCCTCGCAAACC
18S	GATGCGGCGGCGTTATTCC	CTCCTGGTGGTGCCCTTCC
Rat		
F4/80	CTTCAGTGGAACGCATAG	GGTGGCATAAGCTGGACAAGTG
IL-1β	AATGCCTCGTGCTGTCTGACC	GGGTGGGTGTGCCGTCTTTC
Leptin	TTGTCACCAGGATCAATGACATTT	GACAAACTCAGAATGGGGTGAAG
TNF-α	GCAGAGCCTTCCAAGCCTACC	GTTACCCAGCCCACCTCCTTTG
ZO-1	GGAAACCCGAAACTGATGCTATGG	AACTGGCTGGCTGTACTGTGAG
18S	GATGCGGCGGCGTTATTCC	CTCCTGGTGGTGCCCTTCC

R, receptor; IL, interleukin; TNF, tumor necrosis factor; ZO, zona occludens.

Prolong Gold antifading medium (Life Technologies). Serial *x-y* and *x-z* sections were collected every 0.25 μm using a laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Measurement of RhoA activity

RhoA activity was assessed in HT29-19A cell lysates by pull-down assay with the Rho-binding domain of the Rho effector protein rhotekin, as described previously (32, 34). Precipitated active RhoA and total RhoA amounts in cell lysates were analyzed by Western blot using a mouse monoclonal anti-RhoA antibody (Santa Cruz Biotechnologies, CliniSciences, France). Immunoreactive bands were visualized using HRP-conjugated secondary antibody and subsequent ECL detection (GE Healthcare; Velizy-Villacoublay, France).

Statistical analysis

The significance of differences was determined by the Kruskal-Wallis or Mann-Whitney *U* tests, using Prism 3.0 software (GraphPad Software, La Jolla, CA, USA). Differences were considered significant at values of $P < 0.05$.

RESULTS

IUGR leads to visceral adiposity and leaky colon

Rats born with IUGR following maternal protein restriction have a susceptibility to VAT development in adulthood, although fed a normal diet (25, 26). Accordingly, we found that 8-mo-old IUGR rats (n=10) had an increased visceral adiposity, particularly around the cecum and proximal colon, in comparison to control rats (n=10; **Fig. 1*A***). Their visceral fat mass was significantly higher (60%, P<0.01) than in control rats (Fig. 1*B*), in association with higher serum leptin levels (26.5 and 18.9 ng/ml in IUGR and control rats, respectively; P<0.001; Fig. 1*C*). Serum adiponectin was similar in both groups (data not shown). Furthermore, RT-qPCR analysis of VAT collected from IUGR and control rats revealed higher levels of leptin mRNA in VAT of IUGR rats (Fig. 1*D*) but similar mRNA levels of the proinflammatory cytokine TNF-α (Fig. 1*E*) and of macrophage marker F4/80 (Fig. 1*F*). IL-6 and TNF-α concentrations measured by ELISA in VAT lysates were not different between both groups (**Table 2**). Such data suggest that VAT in IUGR rats has no apparent sign of inflammation. Interestingly, the TER of colonic tissues mounted in Ussing chambers was significantly reduced (P<0.05) in the IUGR group as compared to the control group (Fig. 2*A*), suggesting an increased colonic permeability in IUGR rats. Accordingly, serum LPS-binding protein (LBP) concentration, a marker of endotoxemia, was increased in IUGR rats in comparison with controls (3.93±1.8 *vs.* 3.06±0.38 μg/ml, respectively; P<0.05, **Fig. 2*B***). However, Western blot and RT-qPCR analysis of ZO-1 expression in the colon of IUGR and control rats showed similar levels (Fig. 2*C*, *D*). Moreover, no differences in colonic expressions (protein and mRNA) of TNF-α and IL-6 were noticed between the two groups (Table 2 and Fig. 2*E*, *F*). These data indicate that in the rat model of IUGR, colonic visceral adiposity and hyperleptinemia are associated with a leakier colon in the absence of inflammation.

Transplantation of VAT on rat colon increases colonic paracellular permeability by modulating tight junction integrity

Our observation in IUGR rats led us to consider VAT as a potential cause of increased-intestinal permeability. To investigate this possibility, we performed transplantation of VAT from donor rats (with mild visceral adiposity) to the colon of lean recipient rats. Three days after the transplantation, macroscopic and histological examination of the VAT-grafted colons showed

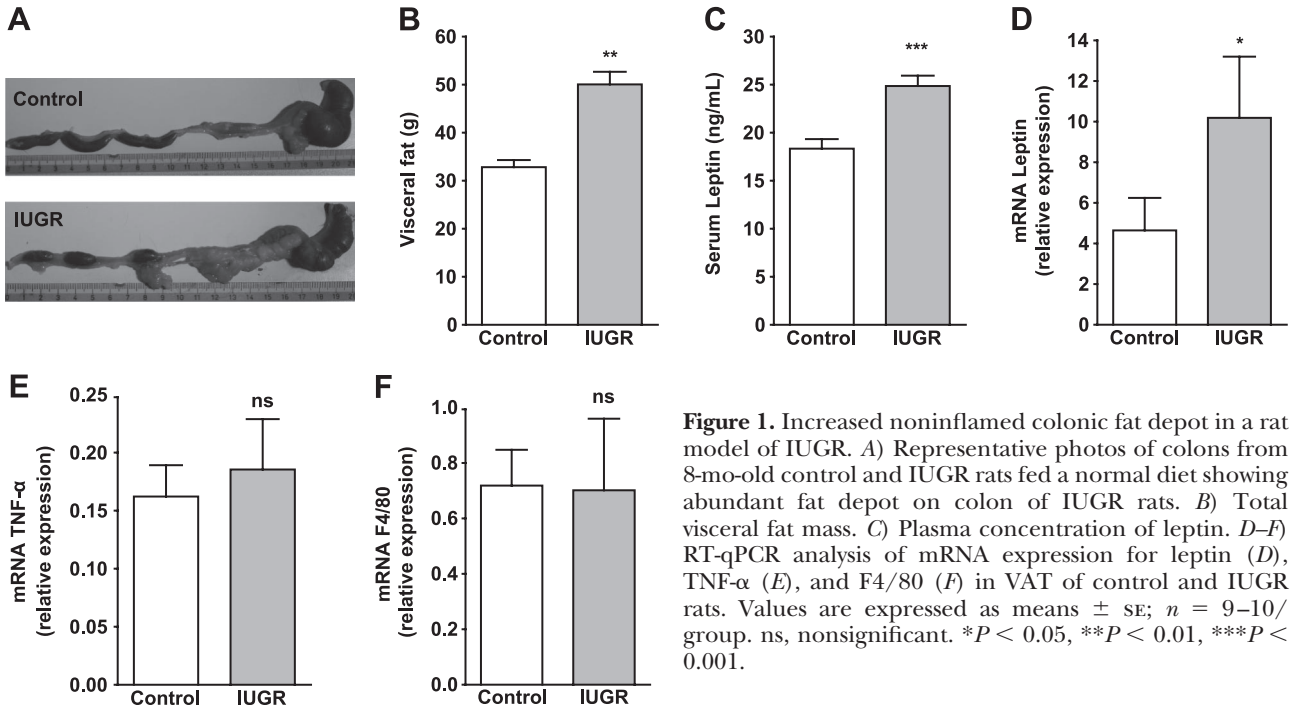

Figure 1. Increased noninflamed colonic fat depot in a rat model of IUGR. *A*) Representative photos of colons from 8-mo-old control and IUGR rats fed a normal diet showing abundant fat depot on colon of IUGR rats. *B*) Total visceral fat mass. *C*) Plasma concentration of leptin. *D–F*) RT-qPCR analysis of mRNA expression for leptin (*D*), TNF-α (*E*), and F4/80 (*F*) in VAT of control and IUGR rats. Values are expressed as means ± SE; $n = 9–10$/group. ns, nonsignificant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

that the graft was viable and well vascularized (**Fig. 3*A***). We found that the colon of VAT-grafted rats had an increased epithelial permeability to FITC-dextran as compared to sham-treated rats (Fig. 3*B*). To assess the consequence of VAT on colonic epithelial barrier function, we quantified serum levels of LBP, a marker of endotoxemia. Serum LBP concentration was increased in rats with VAT grafts in comparison with sham-treated rats (2.08±0.1 μg/ml *vs.* 2.92±0.02 μg/ml; n=6, P<0.05), suggesting an increase in intestinal LPS permeability (Fig. 3*C*). To verify whether the increased epithelial paracellular permeability could involve TJ alterations, we analyzed the expression of the TJ protein, ZO-1. RT-qPCR analysis revealed a strong decrease of mRNA expression in the colon of VAT-grafted rats (Fig. 3*D*). Western blot also showed a decrease ZO-1 protein expression in VAT-grafted rats (Fig. 3*E*). In contrast, no difference was observed in TNF-α and IL-6 protein (ELISA, Fig. 3*F, G*) and mRNA (Fig. 3*H, I*) levels in colonic tissues of grafted and sham-treated rats (Fig. 3*F–I*), suggesting that the increased colonic permeability observed in VAT-grafted rats was not due to local inflammation. This set of data suggests that VAT may directly alter the colonic epithelial barrier function by increasing TJ permeability.

Adipocytes increase TJ permeability of CECs *in vitro*

To investigate the direct role of adipocytes on paracellular permeability of CECs, we performed cocultures of differentiated-PAZ-6 adipocytes with monolayers of Caco-2 cells established on semipermeable filters that allow the diffusion of soluble adipocyte-derived factors. The effect of adipocytes was compared to Caco-2 cells cultured either alone or cocultured with undifferentiated PAZ-6 preadipocytes. In comparison with undifferentiated preadipocytes, PAZ-6 adipocytes had higher levels of leptin and adiponectin mRNA but similar low levels of TNF-α and IL-6 mRNA (**Fig. 4*A***). We measured a fast decrease of the TER of Caco-2 cell monolayers cocultured with differentiated PAZ-6 cells, as compared with Caco-2 cells cultured alone (Fig. 4*B*). Maximal decrease of TER was reached at 5 h and was maintained through the entire duration of culture. Coculture with undifferentiated PAZ-6 cells also induced a decrease in TER of Caco-2 cell monolayer, but the effect was weaker and less sustained than the effect of differentiated PAZ-6 adipocytes. We next examined TJ organization in Caco-2 monolayers by immunofluorescence staining of ZO-1 and occludin.

TABLE 2. *ELISA dosages of cytokine concentration (pg/mg protein) in visceral adipose and colonic tissues of control and IUGR rats*

	IL-6		TNF-α	
Tissue	Control	IUGR	Control	IUGR
VAT	7047 ± 1564	7026 ± 2422 (ns)	465 ± 75	401 ± 162 (ns)
Proximal colon	180 ± 9	233 ± 32 (ns)	16.4 ± 2	15.0 ± 2.4 (ns)

Values are expressed as means ± SE, n = 10. IUGR, intrauterine growth restriction; VAT, vissceral adipose tissue; ns, no significant.

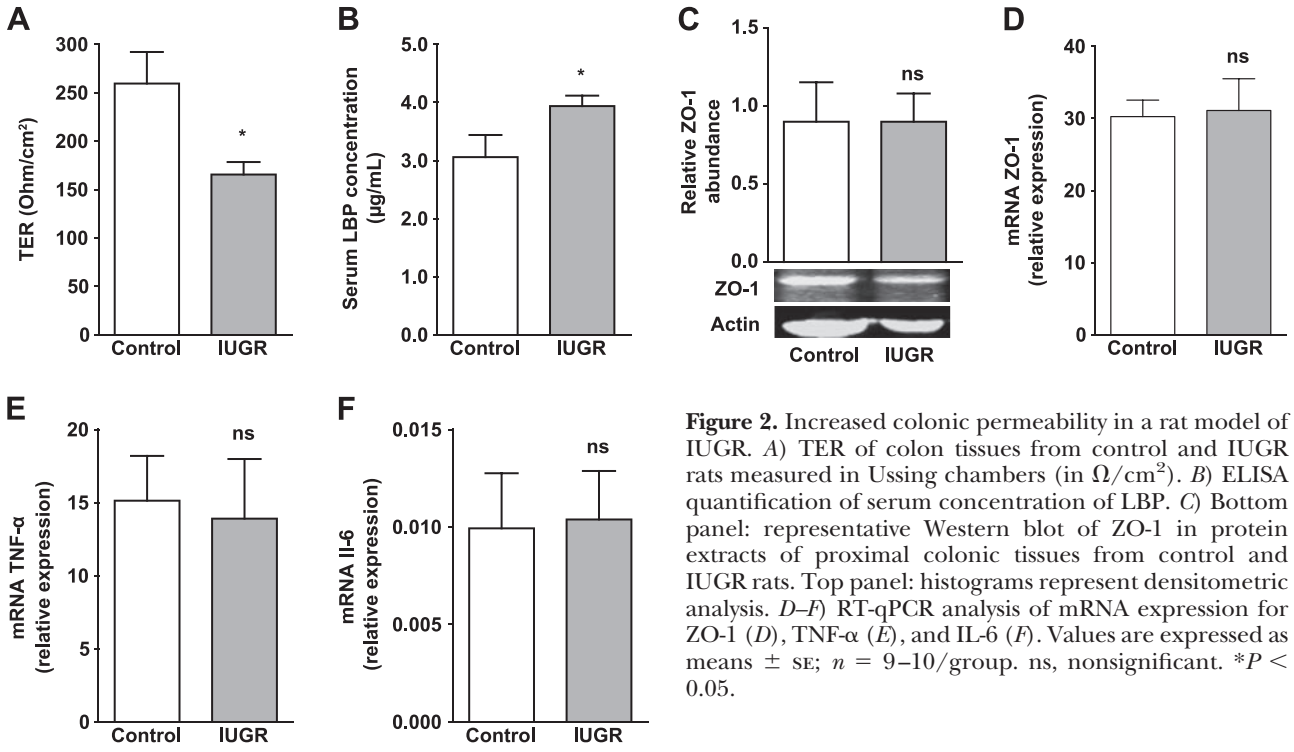

Figure 2. Increased colonic permeability in a rat model of IUGR. *A*) TER of colon tissues from control and IUGR rats measured in Ussing chambers (in Ω/cm^2). *B*) ELISA quantification of serum concentration of LBP. *C*) Bottom panel: representative Western blot of ZO-1 in protein extracts of proximal colonic tissues from control and IUGR rats. Top panel: histograms represent densitometric analysis. *D–F*) RT-qPCR analysis of mRNA expression for ZO-1 (*D*), TNF-α (*E*), and IL-6 (*F*). Values are expressed as means ± SE; $n = 9–10$/group. ns, nonsignificant. $^*P < 0.05$.

Caco-2 cell monolayers cultured alone had a regular distribution of ZO-1 and occludin (Fig. *4C*). Whereas undifferentiated PAZ-6 weakly affect TJ structure (Fig. 4*C*), coculture of Caco-2 cells with differentiated PAZ-6 adipocytes induced a disorganization of cellular junctions with TJ opening as revealed by ZO-1 and occludin immunostaining (Fig. *4C*). These data suggest that soluble adipocyte-derived factors could directly modu-

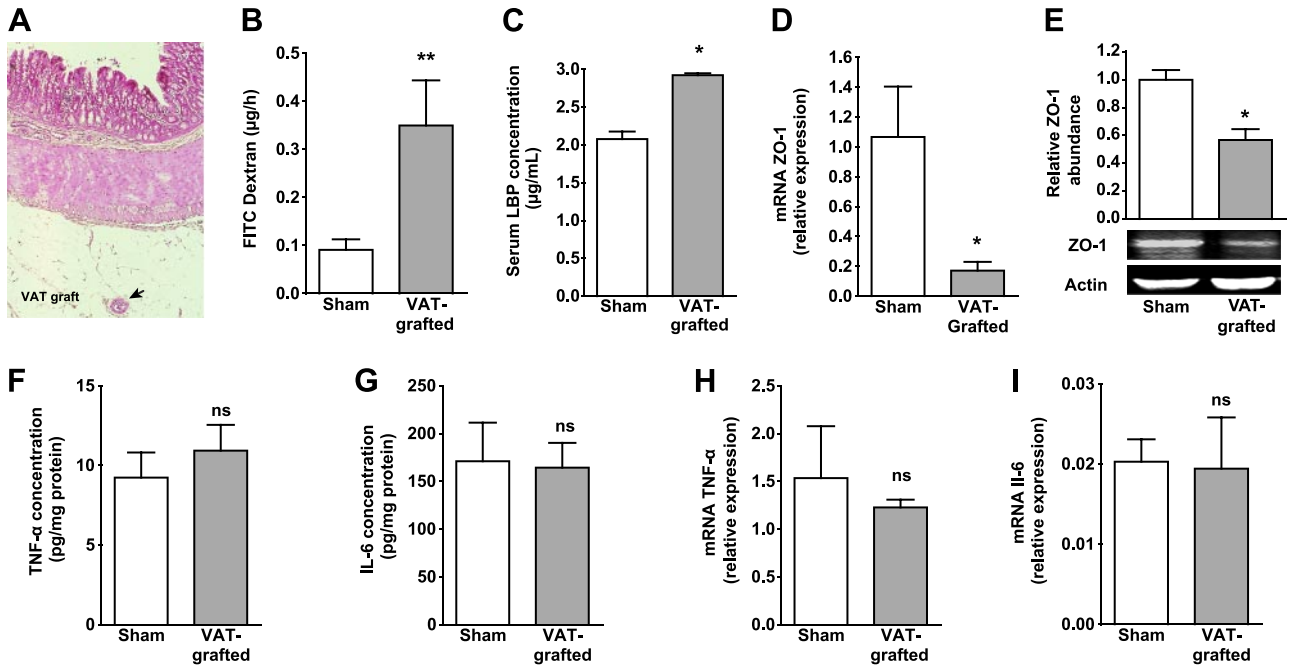

Figure 3. VAT grafts increase colon permeability and alter TJ structure. Lean rats underwent either sham operation or VAT grafting on the colon. *A*) Hematoxylin-and-eosin staining of VAT-grafted colon tissue section. Arrow indicates vessel (view, ×200). *B*) Epithelial paracellular permeability of FITC-dextran measured in colon tissues mounted in Ussing chambers. *C*) ELISA quantification of serum LBP concentration. *D*) RT-qPCR analysis of mRNA expression for ZO-1 in the proximal colon of sham-treated and VAT-grafted rats. *E*) Bottom panel: representative Western blot of ZO-1 in protein extracts of proximal colonic tissues from sham-treated and VAT-grafted rats. Top panel: histograms represent densitometric analysis. *F, G*) ELISA quantification of TNF-α (*F*) and IL-6 (*G*) concentrations in protein extracts of proximal colonic tissues from sham-treated and VAT-grafted rats. Values are expressed as means ± SE; $n = 6$/group. ns, nonsignificant. $^*P < 0.05$, $^{**}P < 0.01$.

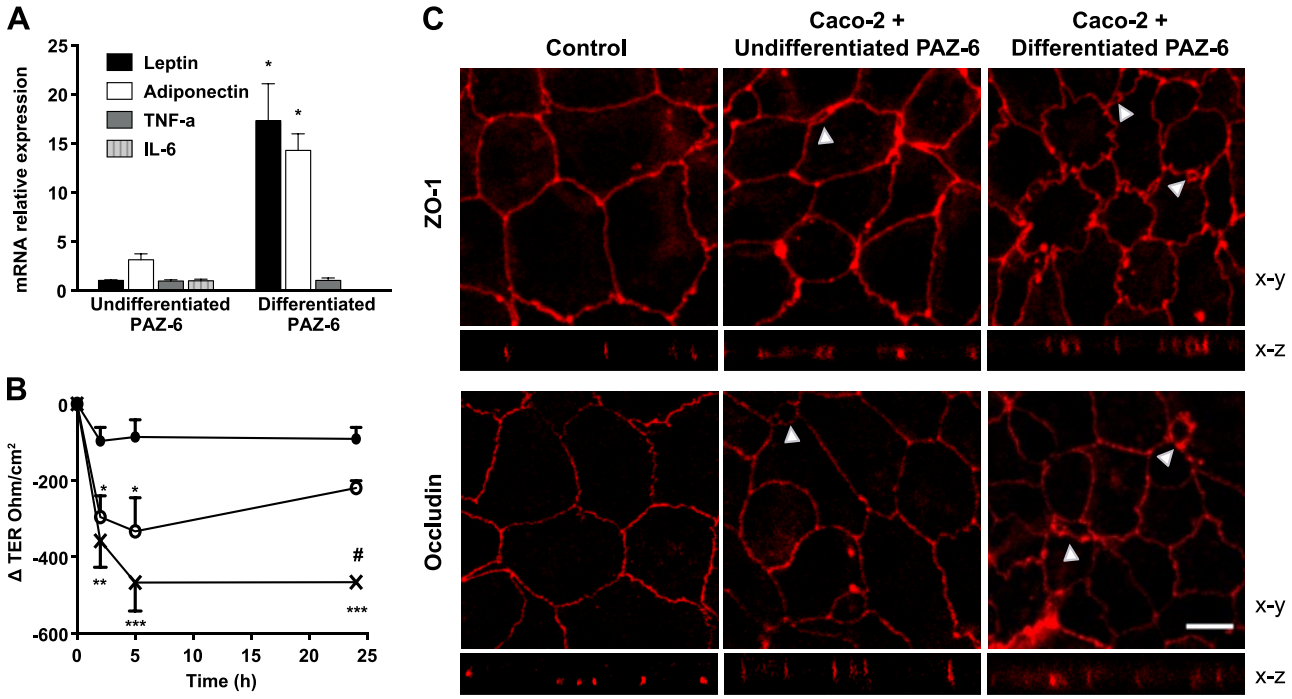

Figure 4. Adipocytes increase paracellular permeability and alter TJ organization in human CECs. *A*) Relative expression of adipokine mRNA in undifferentiated PAZ-6 preadipocytes and differentiated PAZ-6 adipocytes. $^{*}P < 0.05$; $n=3$. *B*) Variations of TER (ΔTER) of Caco-2 cell monolayers cultured alone (solid circles) or cocultured with undifferentiated PAZ-6 cells (open circles) and differentiated PAZ-6 adipocytes (crosses). Values are expressed as means ± SE of the variation (ΔTER) of the initial TER value measured at time 0. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control; $^{\#}P < 0.05$ *vs.* undifferentiated PAZ-6 cells; $n = 5$. *C*) Immunofluorescence staining of the TJ protein ZO-1 and occludin in Caco-2 cell monolayers cultured alone (control), with the undifferentiated PAZ-6 preadipocytes or the differentiated PAZ-6 adipocytes. Confocal microscopy images showing *x–y* and *x–z* axis. Scale bar = 10 μm. Arrows indicate TJ opening.

late paracellular permeability of CECs by opening TJ structures.

Leptin increases intestinal permeability *in vivo*

Among specific adipocyte-derived factors, leptin has been identified as an important modulator of IEC function and a potential mediator of obesity-related colon cancer (35). However, its effect on intestinal epithelial barrier integrity has not been characterized. Therefore, we injected (i.p.) recombinant rat leptin or saline (vehicle) to normal healthy Sprague-Dawley rats and evaluated the effect on intestinal permeability, *in vivo* and *ex vivo*. As compared to vehicle, leptin injection increased the intestinal permeability of the nonabsorbable permeability probe ^{51}Cr-EDTA, which was orally administered and then quantified in rat urine (1.67±0.32 *vs.* 0.73±0.34%; $P<0.05$; $n=10$; **Fig. 5*A***). However, leptin injection in Zucker rats, which have defective leptin receptor, did not increase ^{51}Cr-EDTA in urine (Fig. 5*B*). This result suggests that leptin action on intestinal permeability requires functional leptin receptor. We further analyzed leptin effect in cecum from normal healthy Sprague-Dawley rats mounted in an Ussing chamber. In comparison to vehicle, leptin treatment induced a 4-fold increase in paracellular FITC-dextran flow through colonic tissue (0.085±0.025 *vs.* 0.35±0.13 μg/h; $P<0.05$, $n=6$; Fig. 5*C*). Immunohistofluorescence staining of colon tissue sections showed altered expression and distribution of occludin and claudin-1 in colonic epithelial TJs of rats injected with leptin in comparison to vehicle-injected rats (Fig. *5D*). Taken together, these data suggest that, among specific factors secreted by adipocytes, leptin could be a mediator increasing colonic permeability.

Leptin increases TJ permeability of CECs *in vitro via* a RhoA/ROCK-dependent pathway

Since we observed that leptin affected colonic epithelial permeability and TJs *in vivo*, we next wanted to determine whether leptin could directly act on CECs to alter TJ structure and permeability, *in vitro*. We used HT29-19A CEC monolayers cultured on transwell filters. RT-qPCR analysis showed that HT29-19A cells express mRNA receptors for leptin, adiponectin, IL-6 and TNF-α (data not shown). We tested the effect of these adipokines (10 ng/ml) on HT29-19A cell monolayer integrity. As described previously (4), the proinflammatory cytokines IL-6 and TNF-α led to a rapid decrease in TER and thus served as positive controls (**Fig. 6*A***). However, leptin and adiponectin are more specific adipokines than IL-6 and TNF-α. Interestingly, we found that leptin, but not adiponectin, induced a decrease of TER similar to that of IL-6 and TNF-α (Fig. 6*A*). Leptin-induced TER decrease was dose-dependent

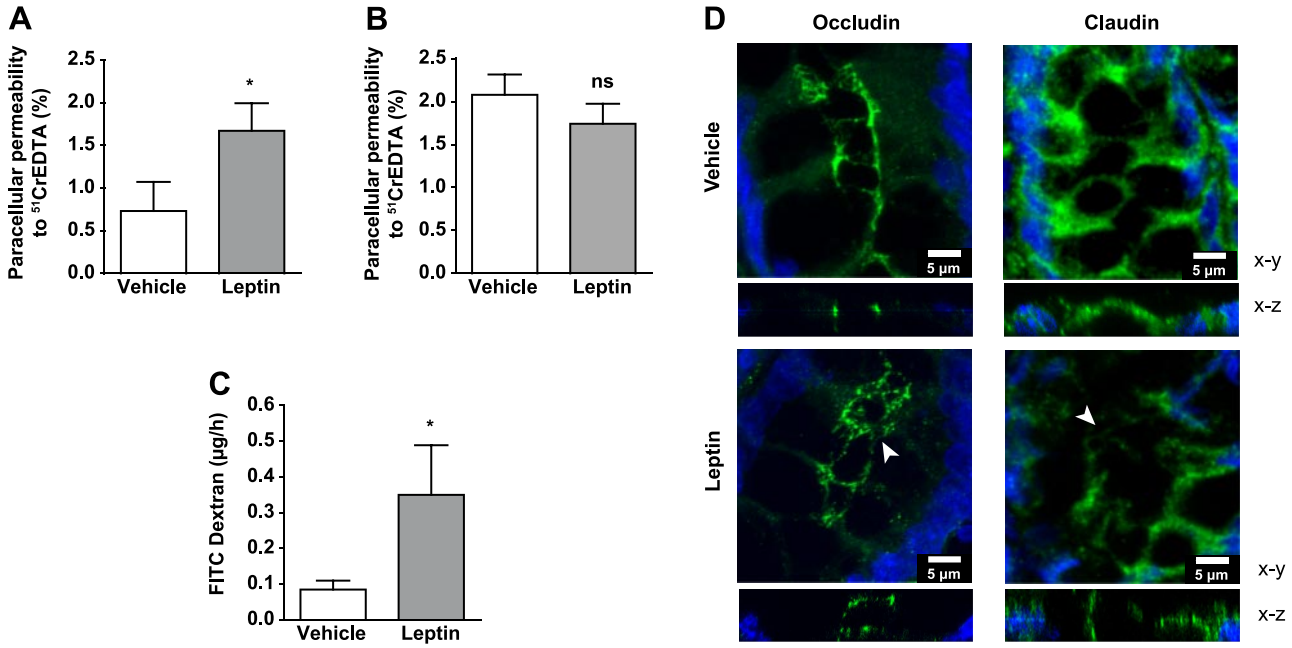

Figure 5. Leptin increases intestinal paracellular permeability and alters TJs *in vivo*. Rats were injected (i.p.) with leptin (50 µg) or vehicle for 6 h. *A*, *B*) Intestinal permeability measured *in vivo* after gavage of Sprague-Dawley (*A*) and Zucker (*B*) rats with ^{51}Cr-EDTA. Data are expressed as the percentage of total radioactivity recovered in urine for 6 h after gavage. *C*) Paracellular epithelial permeability of FITC-dextran measured in colon (cecum) tissues of Sprague-Dawley rats mounted in Ussing chambers. Values are expressed as means ± SE; $n = 6$–10/group. $^*P < 0.05$, ns, nonsignificant. *D*) Confocal microscopy images showing *x*–*y* and *x*–*z* axis of immunofluorescence staining of occludin and claudin in proximal colon tissues. Arrowheads show altered distribution of occludin and claudin in leptin-treated rats. Nuclei are stained with Hoechst.

and was significant at a concentration of 1 ng/ml ($P<0.05$; Fig. 6*B*). Staining of HT29-19A cell monolayer for ZO-1 and F-actin showed that leptin treatment (50 ng/ml) induced a disorganization of both TJ and F-actin cytoskeleton without delocalization of ZO-1 (Fig. *6C*). Because organization of perijunctional F-actin cytoskeleton is regulated by RhoA and its effector ROCK (4), we sought to determine the implication of this pathway in leptin-induced disorganization of the F-actin cytoskeleton. RhoA activity was assessed in cell lysates by pulldown assay. Western blot analysis of the pulldown assay indicated that the amount of active RhoA was increased in leptin-treated HT29-19A cells as compared to untreated control cells (**Fig. 7*A***). Total RhoA amount was equivalent in control and leptin-treated cell lysates. We then analyzed organization of the F-actin cytoskeleton by fluorescence staining with phalloidin. We found that pretreatment of HT29-19A cells with the ROCK inhibitor Y-27632 (10 µM) suppressed leptin-induced disorganization of F-actin cytoskeleton (Fig. *7B*). These data suggest that leptin may directly alter TJ structure and increase paracellular

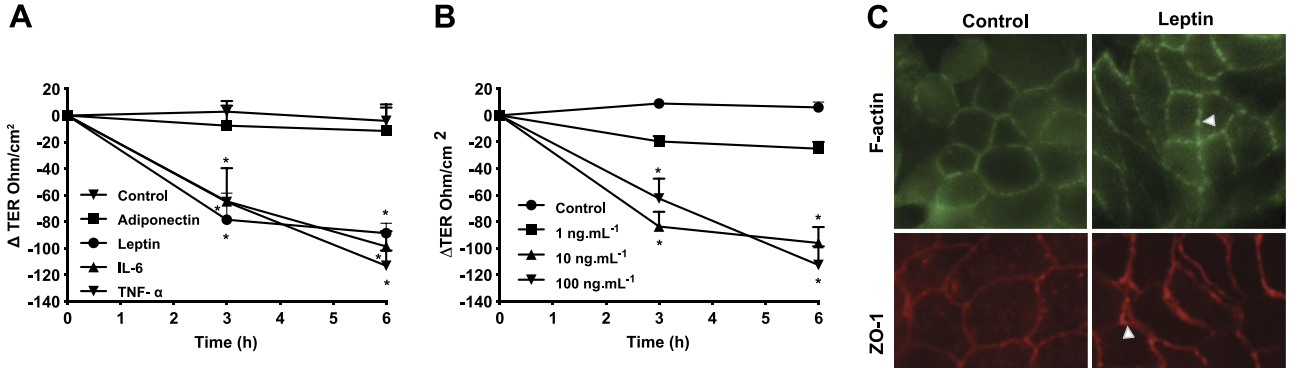

Figure 6. Leptin increases paracellular permeability and alters TJ organization in human CECs. A) Comparison of different adipokines (10 ng/ml) effect and B) dose-response effect of leptin on TER of colonic epithelial HT-29-19A cell monolayers. Values are expressed as means ± SE of the variation (ΔTER) of the initial TER value measured at time 0. $^*P < 0.05$ *vs.* corresponding initial TER value; $n = 6$. *C*) Immunofluorescence staining of the TJ protein ZO-1 and F-actin cytoskeleton in untreated HT29-19A cell monolayer (control) or treated with 50 ng/ml leptin for 6 h. Scale bars = 10 µm. Arrowheads indicate ZO-1 and F-actin disorganization induced by leptin.

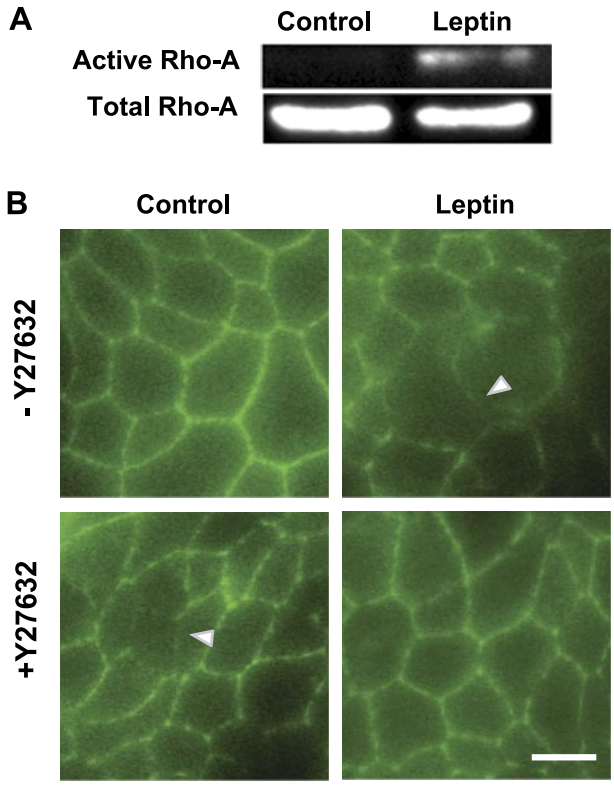

Figure 7. Leptin induces a disorganization of F-actin cytoskeleton through a RhoA/ROCK dependent pathway. *A*) Pull-down assay of cell lysates from untreated (control) and leptin-treated (50 ng/ml) HT29-19A cells for 4 h. Representative Western blot of RhoA showing increased amount of active RhoA in leptin-treated cells. Total RhoA amounts are equivalent between samples. *B*) Immunofluorescence staining of F-actin cytoskeleton in HT29-19A cells untreated (control) or leptin-treated in the absence (−) or presence (+) of the ROCK inhibitor Y27632 (10 μM). Data are representative of 4 independent experiments. Arrowheads show actin cytoskeleton disorganization.

permeability of CECs. This effect of leptin involves disorganization of the perijunctional F-actin cytoskeleton through a RhoA/ROCK-dependent pathway.

DISCUSSION

Excessive development of a VAT surrounding the gastrointestinal tract is found in overweight and obese patients but also in patients with Crohn's disease. There is considerable evidence that VAT-derived adipokines, particularly leptin, are involved in gastrointestinal pathologies, including obesity-related colon cancer and IBD (13, 18, 35). However, most human and animal studies have considered the role of VAT as a consequence of the disease process *per se*. Recently, a positive association was reported between intestinal permeability and visceral adiposity in normal to mildly overweight otherwise healthy women that had no history of, and were free from gastrointestinal disease (24). A progressive increase in visceral adiposity is also observed with aging but the role of VAT in induction of early modifications of the intestinal epithelium has been poorly investigated. Thus, in the present study, we sought to investigate the possibility of a direct role of VAT and adipokines on gut permeability. We show for the first time that VAT and, specifically, the adipocyte *per se* may be causally linked to increased colonic epithelial permeability, independently of any context of obesity or inflammation. Among adipokines, we show that leptin may initiate gut leakage and that leptin-induced epithelial TJ-permeability involves disorganization of the perijunctional F-actin cytoskeleton *via* a RhoA-ROCK-dependent pathway.

Growing evidence suggests that a poor fetal growth favors the developmental programming of increased visceral fat (36). In this study, we have used a model of rat born with an IUGR, a condition that promotes proliferation of preadipocytes and the development of visceral adiposity in adulthood, even when fed a standard chow (25, 26). In this model, a trancriptomic analysis of VAT showed an up-regulation of genes involved in lipid metabolism, but in contrast with rodent models of obesity, a down-regulation of several genes associated with inflammation (25). In accordance, we found that levels of proinflammatory cytokines or of the macrophage marker F4/80 are not elevated in VAT of IUGR rats as compared to controls, suggesting that adipose tissue in IUGR rats is not inflamed. In addition, increased visceral adiposity in IUGR rats correlates with higher leptin mRNA and serum leptin levels. Thus, together with previous reports (25), our data suggest that, in IUGR, adipocytes may play a more important role than cells of the stromal vascular fraction. Of importance, IUGR rats display an increased colonic permeability without apparent signs of inflammation in the colon. Similarly, we previously reported the absence of inflammation in the jejunum of IUGR rats (27). We, therefore, hypothesized that adipokines secreted by VAT surrounding the colon could partly explain the loss of intestinal barrier integrity. We demonstrate that a graft of VAT on the colon of lean recipient rats induces an increase in intestinal paracellular permeability that is associated with altered expression and distribution of the TJ proteins ZO-1 and occludin. This effect does not seem to be a consequence of an inflammatory response to surgery, since colonic tissues of both VAT-grafted and sham-treated rats have similar levels of TNF-α and IL-6 mRNA. However, adipose tissue is composed of different cell types, including adipocytes, preadipocytes, endothelial cells, and immune cells that can secrete multiple mediators acting through endocrine, autocrine, and paracrine pathways (16, 17). We demonstrate that, *in vitro*, differentiated PAZ-6 adipocytes can directly induce an increase of CEC permeability and alter ZO-1/TJ structure. Interestingly, differentiation of PAZ-6 adipocytes is characterized by an increased expression of mRNA for the adipokines leptin and adiponectin but not for inflammatory cytokines TNF-α and IL-6 as compared to undifferentiated PAZ-6 preadipocytes. Therefore, the larger effect of differentiated

adipocytes on CEC permeability in comparison to preadipocytes suggests that adipokines rather than cytokines could be involved in the increase of CEC permeability.

Notably, in our study, the fact that the colon in IUGR rats or in VAT-grafted rats is not inflamed constitutes a striking difference with genetic and dietary models of obesity (9–12). In the latter models, it is suggested that gut inflammation is a leading cause of TJ disorganization and consequently of increased intestinal permeability to the bacterial product LPS that, in turn, drives visceral fat inflammation and metabolic dysfunction. The role of gut inflammation in VAT dysfunction is supported by data showing that in both obesity and Crohn's disease, VAT displays similar metabolic dysfunctions and inflammatory cytokine profile (12, 37). As a consequence, it is suggested that VAT-infiltrating macrophages secrete inflammatory products that contribute to amplification and perpetuation of gut inflammation. Our study provides new information by suggesting that the development of a normal, noninflamed, visceral adipose tissue could constitute an early event inducing alteration of the epithelial barrier. An early increase of intestinal permeability would, in turn, allow the entry of bacterial products, such as LPS that may trigger inflammatory responses, thus leading to a vicious circle as described by Batra *et al.* (38), where inflammatory conditions in the intestine and VAT support each other. Our data also suggest that VAT-induced gut leakage in IUGR could lead to a "metabolic endotoxemia" (10), and represent an early step in the process leading to the metabolic dysfunctions associated with IUGR (39). However, as hypothesized by Waterland (40), we cannot exclude that IUGR also induced a fetal programming affecting gut physiology, in particular, epithelial barrier function. Indeed, it has been described that IUGR rats have alterations of mucin expression and intestinal microbiota composition (41). Furthermore, we previously reported that jejunum from IUGR rats have a reduced expression of intestinal alkaline phosphatase in response to a high-fat diet (27). Longitudinal studies are needed to determine the temporal interactions between IECs and adipocytes in the context of gut inflammation and permeability.

Our study identifies leptin as a key mediator of adipocyte-induced increase of CEC permeability. We show that i.p. injection of leptin to lean rats induces an increase in colonic epithelial permeability, an effect supported by disorganization of TJ structure. Leptin receptor is expressed in both apical and basolateral membranes of epithelial cells of small intestine and colon (20–22), suggesting that leptin may be involved in the physiological regulation of epithelial cell function. *In vivo*, its role in regulating epithelial cell proliferation, mucin secretion or intestinal glucose absorption has been described previously (22, 23, 42). More recently, a proteomic analysis of leptin effect on mouse colon showed that leptin regulates the expression of proteins involved various cell functions, including energy metabolism and cell proliferation (43). However, chronic exposure of colonic epithelium to leptin secreted by locally developed VAT may lead to pathological consequences as suggested in obesity-related colon cancer or IBD (14, 18, 35). Indeed, *in vitro* studies have shown that leptin stimulates cell proliferation, inhibits apoptosis, and stimulates migration and invasiveness of colon cancer cell lines (44–46). Our study provides evidence for a new function for leptin on intestinal barrier integrity. Disorganization of TJ integrity by leptin could also participate to an early loss of epithelial cell polarity that can be relevant to colon carcinogenesis and to gut-VAT interaction in modulating gut inflammation.

However, in genetic obese *ob/ob* and *db/db* mice, increased intestinal permeability is observed, although leptin function is lost (9). In genetic models of established obesity, the increased intestinal permeability seems to rather correlate to the increased circulating levels of inflammatory cytokines such as INF-γ, TNF-α, and IL-1β, known major markers of the low-grade inflammatory phenotype. Also, we cannot exclude that *in vivo* injection of leptin may stimulate the release of proinflammatory cytokines by different cell types, including immune cells and thus indirectly increase intestinal permeability. However, we demonstrate here for the first time that leptin, but not adiponectin, directly acts on CECs to increase paracellular permeability, *in vitro*. The effect of leptin is comparable to that of TNF-α, a proinflammatory cytokine well known to increase epithelial paracellular permeability (4, 8). We show that leptin activates RhoA and its effector ROCK in CECs leading to disorganization of perijunctional F-actin cytoskeleton without delocalization of ZO-1. These data suggest that leptin could induce TJ opening through ROCK-mediated contraction of the perijunctional actomyosin ring (4). Activation of the RhoA/ROCK pathway is involved in TJ disruption by several pathogenic and inflammatory stimuli, such as TNF-α (4, 47). *In vitro* studies have also reported that leptin stimulation of the RhoA/ROCK pathway is involved in invasiveness of CECs (45) and in vascular smooth muscle hypertrophy (48). Leptin activation of RhoA/ROCK may also be relevant to intestinal inflammation since it has been shown that this pathway stimulates the transcription factor NF-κB in IBD (34). Collectively, these data comfort the role of leptin in gut barrier impairment that would involve the activation of RhoA/ROCK leading to TJ opening .

In summary, we provide new insight into the role of VAT, which is considered here as an early factor inducing impairment of colonic epithelial barrier. The early and chronic paracrine action of leptin on colonic epithelium could lead to TJ disorganization and enhanced permeability to bacterial LPS. Thus, VAT and leptin may, in turn, initiate a low-grade inflammatory state that precedes metabolic dysfunction of other organs. FJ

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Loss of Vagal Sensitivity to Cholecystokinin in Rats Born with Intrauterine Growth Retardation and Consequence on Food Intake

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Perinatal malnutrition is associated with low birth weight and an increased risk of developing metabolic syndrome in adulthood. Modification of food intake (FI) regulation was observed in adult rats born with intrauterine growth retardation induced by maternal dietary protein restriction during gestation and maintained restricted until weaning. Gastrointestinal peptides and particularly cholecystokinin (CCK) play a major role in short-term regulation of FI by relaying digestive signals to the hindbrain *via* the vagal afferent nerve (VAN). We hypothesized that vagal sensitivity to CCK could be affected in rats suffering from undernutrition [low protein (LP)] during fetal and postnatal life, leading to an altered gut–brain communication and impacting satiation. Our aim was to study short-term FI along with signals of appetite and satiation in adult LP rats compared to control rats. The dose–response to CCK injection was investigated on FI as well as the associated signaling pathways activated in nodose ganglia. We showed that LP rats have a reduced first-meal satiety ratio after a fasting period associated to a higher postprandial plasmatic CCK release, a reduced sensitivity to CCK when injected at low concentration and a reduced presence of CCK-1 receptor in nodose ganglia. Accordingly, the lower basal and CCK-induced phosphorylation of calcium/calmodulin-dependent protein kinase in nodose ganglia of LP rats could reflect an under-expressed vanilloid family of transient receptor potential cation channels on VAN. Altogether, the present data demonstrated a reduced vagal sensitivity to CCK in LP rats at adulthood, which could contribute to deregulation of FI reported in this model.

Keywords: perinatal malnutrition, gastrointestinal peptide, nodose ganglia, satiation, CCK signaling

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INTRODUCTION

Metabolic pathologies such as obesity and type 2 diabetes are a worldwide public health problem especially in Western countries. Although life style, decreased physical activity and nutritional transition are the main causes of predisposition to obesity, a large body of epidemiological studies linked a low birth weight, consequence of intrauterine growth retardation (IUGR), to a higher

Abbreviations: BW, bodyweight; CART, cocaine amphetamine-regulated peptide; CaMKII, calcium/calmodulin-dependent protein kinase; CCK, cholecystokinin; CCK-1R, CCK-1 receptor; ERK, extracellular signal-regulated kinase; FI, food intake; GIP, glucose-dependent insulinotropic peptide; I.P., intraperitoneal; IUGR, intrauterine growth retardation; LP, low protein; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; PYY, peptide YY; TRPV, vanilloid family of transient receptor potential; VAN, vagal afferent nerve; Y2-R, Y2 receptor.

risk of metabolic pathologies at adulthood. It is indeed well documented that low birth-weight babies present a higher susceptibility to develop obesity, insulin resistance, cardiovascular diseases, and type 2 diabetes later in life (1–3). More generally the metabolic programming concept proposes that a deleterious nutritional environment inflicted during fetal and early postnatal life could impact long-term health (4).

Animal studies have demonstrated that perinatal undernutrition may lead to a programmed hyperphagia that in the long term led to adult obesity (5). Previous studies in our laboratory demonstrated that low-protein (LP) rats eat more of a regular chow diet from weaning to 2-month old (6, 7). At adulthood despite a higher speed of ingestion (6, 8), a delay in appearance of satiety (satiation) is still observed with more food consumed during the first meal (7). LP rats have also a greater appetite for high-energy diet at adulthood (8) and a preference for high-fat food in the offspring of undernourished dams has also been reported (9), which could contribute to an increase of the body weight during adulthood and later on to obesity.

Food intake (FI) is a highly integrated behavior that relies on complex interactions between neuronal populations located in hypothalamic nuclei, brainstem, and cerebral nuclei implicated in hedonism, motivation, and activation of the peripheral autonomous system. In the central nervous system, the arcuate nucleus contains two well-characterized neuronal populations that act with opposite effects on feeding. Anorexigenic proopiomelanocortin neurons synthetize α-melanocyte-stimulating hormone and cocaine and amphetamine-regulated peptide (CART) whereas orexigenic neurons express neuropeptide Y (NPY) and agouti-related protein (10).

Animal studies have shown that altered maternal nutrition disturbs this hypothalamic system in offspring in favor of orexigenic activity predisposing to hyperphagia (6, 11). Long-term mechanisms of FI regulation by peripheral signaling (leptin, insulin) that monitor energy stores and availability to maintain homeostasis are particularly sensitive to perinatal nutrition. In LP rats, impaired leptin and insulin signaling in arcuate nucleus has been demonstrated to contribute to hyperphagia (12). By contrast, the short-term mechanisms of FI, which regulate on one hand anticipated appetite through ghrelin action and on the other hand meal size and the inter-meal time through peptides from the digestive tract have been poorly studied in maternal food-deprived offspring.

Short-term regulation of FI is controlled by the integration of digestive signals by the vagus nerve into the nucleus of the solitary tract (NTS) in the hindbrain that initiates satiation by a vago-vagal reflex (13). Among these signals, gastrointestinal peptides and mainly cholecystokinin (CCK) are key regulator of short-term FI (14). Vagal afferent nerves (VANs) are primary target of CCK and it is now well demonstrated that they represent a major site for integration of peripheral signals controlling FI (15). Indeed, CCK-1 receptors (CCK-1R) are expressed in nodose ganglia and CCK release after a meal stimulates expression of Y2 receptor (Y2-R), which responds to the anorexigenic gut peptide YY (PYY) and the release of CART in VAN while expression of the orexigenic peptide melanin-concentrating hormone (MCH) is suppressed. By contrast, during fasting, when plasma CCK concentration is low, MCH receptor expression increases while Y2-R and CART expression is reduced (16). As a gatekeeper, CCK operates this vagal neurochemical phenotype switch according to the energy state (14). Interestingly, this normal switching between feeding and fasting states is blunted in diet-induced obesity rats as demonstrated by a loss of vagal sensitivity to leptin (17), which is known to act synergistically with CCK on VAN to potentiate the satiety effect of CCK (18).

Cholecystokinin suppression of FI involves numerous signaling pathways. In NTS neurons, CCK activity involves extracellular signal-regulated kinase (ERK) signaling cascade (19–21). In nodose neurons, CCK induces an increase in intracellular calcium mediated by members of the vanilloid family of transient receptor potential (TRPV)2–5 cation channels (22). Transient calcium signal is converted by calcium/calmodulin-dependent protein kinase (CaMKII), which autophosphorylates and functions as an intracellular signaling element. Prolonged phosphorylation of CaMKII reflects cellular activation (23).

We hypothesized that in LP rats, alteration of short-term FI and particularly satiation could be due to a loss of vagal sensitivity to CCK and/or an alteration of the vagal phenotype leading to a compromised integration of short-term satiety signals. Therefore, the aim of the present study was to investigate the reactivity to CCK in the adult offspring of dams fed a control or a LP diet during lactation and gestation. First-meal pattern following fasting was studied in physiological cages and plasma concentrations of various gastrointestinal peptides were measured in pre- and postprandial period. A dose–response to intraperitoneal (i.p.) CCK agonist (CCK-8S) injection was performed to quantify CCK sensitivity by measuring FI. Neurochemical vagal phenotype was determined in fasting/feeding states and vagal activation following CCK injection was analyzed by measuring phosphorylation of CaMKII and ERK in nodose ganglia.

ANIMALS AND METHODS

Animals

Pregnant females (gestation day 1) were obtained from Janvier (Le Genest Saint Isle, France), housed individually under standard laboratory conditions with free access to either a control (20% w/w protein, $n = 8$) or an isocaloric LP diet (8% protein, $n = 8$) through gestation and lactation. Both diets were purchased from AB diets (Woerden, The Netherlands) and composition is provided in Table S1 in Supplementary Material. At delivery, litter size was adjusted to eight male pups per dam. Pups were pooled and randomly attributed to foster mothers to create two experimental groups. Pups born from control dams were adopted by foster control ones (control, $n = 24$) and pups born from LP dams were attributed to foster LP dams (LP, $n = 24$). Control and LP offspring were weaned at 21 days of age and received a standard laboratory chow (A04, Safe, Augy, France) until adulthood. As previously described in this model (6, 24), birth weight of LP rats was 7–9% lower than control birth weight and this difference persisted until adulthood (130–160 days).

First-Meal Pattern Analysis

At 160 days of age, rats (8–12 animals/group) were individually housed (22 ± 2°C, 12:12-h light/dark cycle, lights on at 7:30)

for 3 days in plexiglas Phecomb cages (Bioseb, Vitrol, France) equipped to monitor meal pattern by continuous and automatic weighing of food. Phecomb system weights the food tray for each second. It allows precisely quantifying the food consumption and identifying feeding events. Artifacts as large vibrations when the rat enters in contact with the tray but without eating are taken into account by filters on the hardware and the software (Phecomb system monitoring software Compulse). A percentage of reliability of the quality signal was calculated by the software and only experiments with a percentage >80% have been used. A meal was defined with a minimal size of 0.5 g and a minimum inter-meal interval of 20 min. A meal is composed of several bouts. Meal parameters extracted from Compulse software included latency to eat, meal size, duration of the meal, inter-meal interval, and satiety ratio. After 48 h of fasting and 24 h of acclimatization to the cage, data were recorded from the beginning of the second day (8:00 a.m.) each 5 s over a 24-h period. This prolonged caloric restriction of 48 h was chosen on the basis of previous reports showing that 24–48 h duration of fasting triggers VAN to switch from anorectic to orexigenic phenotype (16, 25, 26). Other previous studies used this duration of fasting to promote feeding in adult rats weighting more than 500 g (6, 7).

Fasting-Feeding Experiment

Control ($n = 16$) and LP rats ($n = 16$) were fasted for 48 h (water *ad libitum*) and then divided into two subgroups: one group not refed and the second group refed during 2 h. Rats were killed by CO_2 inhalation and cervical dislocation. Stomach, duodenum, ileum, and nodose ganglia were rapidly dissected and collected for further analysis. Portal blood sample was collected in EDTA-containing tubes (Coveto, Montaigu, France). To preserve active form of gastrointestinal peptides, portal blood (200 µL) was collected in less than 30 s and directly flushed within EDTA tubes containing a mix of protease inhibitors including dipeptidyl peptidase IV inhibitor (diprotin A 100 µM, Sigma, Saint Louis, MO, USA), serine protease inhibitors (aprotinin, approx. 400 TIU/L, Sigma), and protease cocktail inhibitors (diluted 1/100, Sigma). After centrifugation, plasma (50 µL) was then aliquoted in microtube containing the same mix of protease inhibitors and immediately frozen at −80°C.

Plasma Gastrointestinal Peptides

Plasma concentration of rat non-sulfated CCK-8 (CCK-8NS) and desacylated ghrelin was analyzed by ELISA (EIA kit, Phoenix France, Strasbourg and SpiBio, Montigny le Bretonneux, France, respectively). Plasma total glucose-dependent insulinotropic peptide (GIP) and total PYY were assayed by Milliplex mag (Rat metabolic magnetic bead panel kit, Millipore, MA, USA).

Kinetics of CCK Release

Rats ($n = 9$–12 animals/group) were fasted for 15 h and refed (chow) at the beginning of the dark phase. This duration of fasting was chosen to induce hunger in adult rats while limiting leptin deficiency that attenuated response to meal-related satiety signals (27). Their FI was measured by food tray weighing at 60, 90, and 150 min post-refeeding. Concomitantly, blood was collected from the tail vein in EDTA-containing tubes (Microvette CB300 EDTA 3K, Sarstedt, Marnay, France) at 0 (15 min before refeeding), 15, 30, 60, 90, and 150 min after the beginning of the meal.

Sensitivity to CCK

Rats were deprived of food during 15 h and were intraperitoneally injected with either sterile NaCl 0.9% (saline) or CCK octapeptide, sulfated (CCK-8S, Bachem, Germany) just before light off and refeeding. FI and spillage were weighed every 30 min during 90 min. The satiating effect of CCK-8S was tested on distinct animals, for three doses [0.25, 2.5, and 7.5 nmol/kg of bodyweight (BW)] vs vehicle (saline) on consecutive days. It means that each rat received one of the treatment on a given test day followed by a period of 3–5 days of wash out and resting between each injection. Therefore, the whole experiment extended over 3 weeks. Doses of CCK were chosen on the basis of previous published data. The low dose (0.25 nmol/kg BW) reduces 1-h FI by 25% in standard fed rats (28). The 10-fold higher dose (2.5 nmol/kg BW) is necessary to induce FI reduction in diet-induced obesity rats (29) and the higher dose (7.5 nmol/kg BW) is used to induce anorectic effect in $MC4R^{-/-}$ obese rats (30).

CCK-Vagal Activation

Rats were fasted for 15 h and then received an intraperitoneal (i.p.) injection with either CCK-8S (0.25 nmol/kg BW) or saline 5 min before refeeding and light off. Twenty minutes after injections, rats were killed by CO_2 inhalation followed by cervical dislocation and nodose ganglia were rapidly collected for Western blot analysis.

Western Blot Analysis

Nodose ganglia were homogenized at 4°C in RIPA lysis and extraction buffer as previously described (31). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Marnes la Coquette, France). Fifteen micrograms of protein was solubilized in electrophoresis sample buffer, loaded in ready-to-use (4–15%) polyacrylamide gels (Mini-protean TGX, Bio-Rad) and transferred onto Trans-Blot Turbo membrane (Bio-Rad). Protein was probed with anti-pCaMKII mouse polyclonal antibody (diluted 1/2,000, pT286, Thermo Scientific) and anti-CaMKII rabbit monoclonal antibody (diluted 1/1,000, Abcam, France). Following de-hybridization, membrane was probed with rabbit polyclonal anti-pERK and total ERK antibodies (diluted 1/1,000, Abcam). Immunoreactive bands were visualized with DyLight™680- and 800-conjugated antibodies, respectively (KPL, Eurobio, France). Band intensities were quantified by infrared scanning densitometry (Odyssey Imaging Systems, LI-COR, Germany). Data are expressed as the ratio of phosphorylated protein relative to total protein. Protein load was controlled with anti-actin mouse monoclonal antibody (diluted 1/2,000, Sigma).

Immunohistochemistry

Cryostat sections (7 µm) of fixed nodose ganglia in 4% paraformaldehyde were mounted on SuperFrost Plus Gold slides (Thermo Scientific; Braunschweig, Germany). Sections were stained with a rabbit polyclonal antibody raised against CCK-1R diluted at 1/200 (Santa Cruz Biotechnology, CA, USA). The secondary antibody

used at appropriate dilution (1/1,000) was a goat biotinylated anti-rabbit polyclonal antibody (Vector Laboratories, Clinisciences, Nanterre, France) followed by Alexa Fluor® 488-conjugated streptavidin antibody (Molecular Probes, Life Technologies). Nuclei were counterstained with DAPI. Tissues sections were mounted in Prolong Gold antifading medium (Molecular Probes, Thermo Scientific, Courtaboeuf, France). Three to five sections of nodose ganglia per rat were analyzed by fluorescence microscopy (Zeiss Axiovert 200M, Carl Zeiss, France). Fluorescence was quantified on image mosaic representing the total surface of each longitudinal nodose ganglia section using Volocity software (Volocity 6.2.1, Perkin Elmer, France).

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated with TRIzol reagent (Invitrogen, Life Technologies) and treated for 45 min at 37°C with RQ1 DNAse (Promega, Charbonnières-Les-Bains, France). One microgram RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen). Five microliters of a 1/20 (nodose ganglia) or 1/40 (intestinal tissue) dilution of cDNA solution were subjected to RT-qPCR in a Bio-Rad iCycler iQ system using the qPCR SYBR Green MasterMix (Fermentas, Courtaboeuf, France). Quantitative PCR consisted of 45 cycles, 30 s at 95°C and 30 s at 60°C each. Primers sequences are figured in Table S2 in Supplementary Material.

Statistical Analysis

All statistical analyses were carried out using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) software. Comparisons between control and LP groups were performed with an unpaired, two-tailed, Mann–Whitney test. When measures were repeated (dose–response), Friedman test was applied following Wilcoxon signed rank test. A P value ≤ 0.05 was considered statistically significant.

RESULTS

First-Meal Pattern and Plasma Gastrointestinal Peptides

Following 48-h fasting, first-meal size (grams per kilogram BW) was significantly higher in LP rats and the inter-meal interval preceding the second meal tended to be reduced, leading to a significant 30%-lower satiety ratio in LP rats as compared to the control group (**Figure 1**). Since these short-term parameters of FI are primarily regulated by gastrointestinal peptides, gene expression (Figure S1 in Supplementary Material) and plasma concentration of ghrelin (stomach), CCK, GIP (duodenum), and PYY (ileum) were performed in fasted and 2 h-refed control and LP rats (**Figure 2**). As expected, plasma desacylghrelin was significantly decreased 2 h post-feeding but there were no differences between control and LP rats. Basal plasma concentration of CCK-8NS was similar in the both groups suggesting no IUGR effect on CCK production by I-cells. By contrast, postprandial CCK in LP rats was significantly higher than basal concentration 2 h post-feeding whereas in control rats CCK concentration reached back the initial basal level. This result supports the hypothesis of an alteration in the postprandial feedback regulation of CCK secretion and could be related to failure in short-term mechanisms of FI observed in LP rats (enhanced meal size and reduced satiety ratio). Plasma concentration of GIP and PYY (secreted further distally in the gastrointestinal tract than CCK and GIP) were significantly higher 2 h post-feeding as compared to fasting but with no difference between control and LP groups. Neither basal nor postprandial gene expression of these gastrointestinal peptides was affected by perinatal LP diet (Figure S1 in Supplementary Material).

Since plasma CCK concentration remained higher than basal concentration 2 h post-feeding in LP rats, we sought for an alteration in the kinetic of production of CCK over the feeding period. Plasma concentration of CCK was measured from 15 min before and until 150 min after feeding. As shown in **Figure 3A**, CCK release in response to feeding was similar in both groups but from 1 h to 2 h 30 min after FI plasma concentration of CCK was significantly higher in LP rats as compared to control rats. While in control group CCK concentration reached basal value as soon as 60 min post-feeding, values in LP rats were still higher than basal concentration at 150 min post-feeding. The total release of plasma CCK evaluated by the area under curve over the completed period (0–150 min) was significantly higher in LP rats as compared to control rats (**Figure 3A**). These results clearly showed an alteration in the regulation of postprandial CCK release in LP rats.

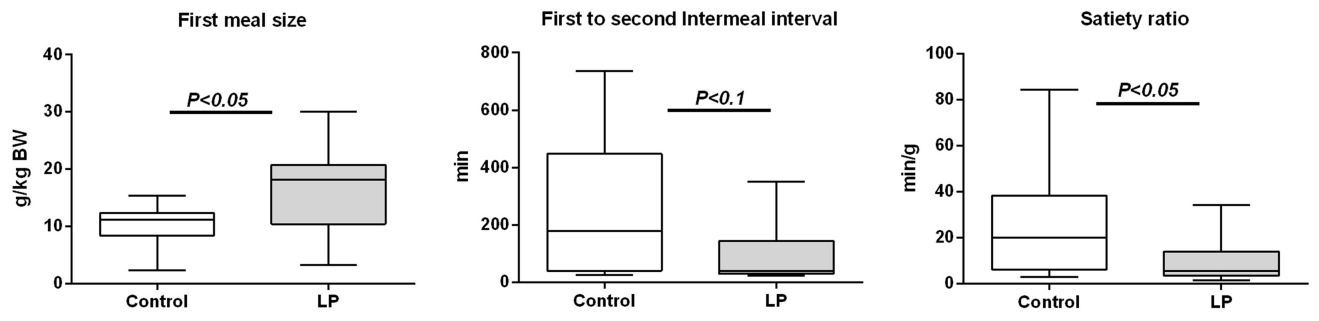

FIGURE 1 | First-meal pattern measured in physiological cages after 48-h fasting in 160-day-old control [n = 12; 631 ± 67 g bodyweight (BW), open bars] and low-protein (LP) (n = 10; 551 ± 31 g BW, gray bars) rats (BWs are means ± SD). Animals were refed *ad libitum* at 8:00 a.m. and meal pattern was recorded during 24 h. Data are plotted as min to max values in box and whisker with $P < 0.05$ considered as significant difference between the two groups (Mann–Whitney test).

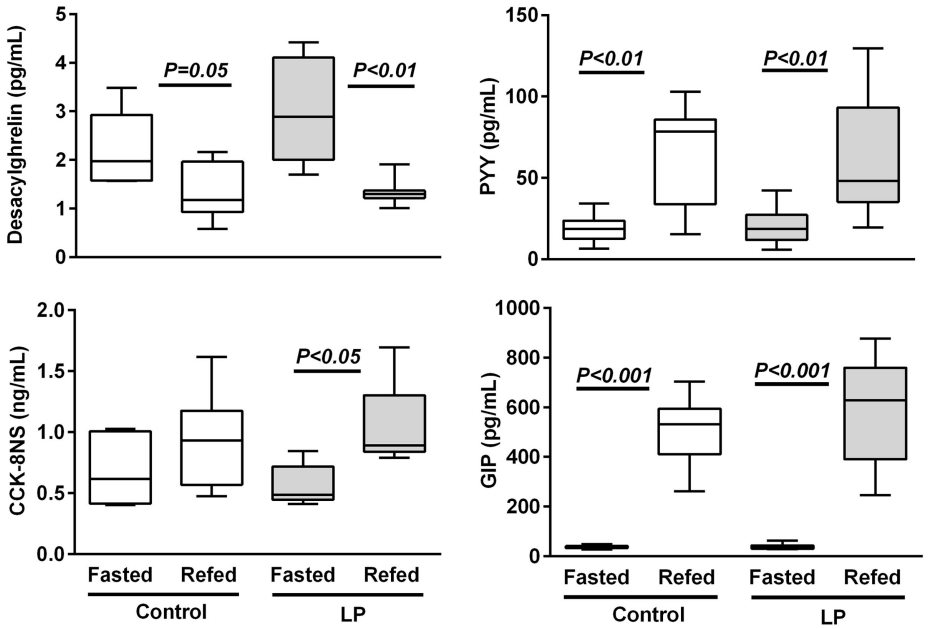

FIGURE 2 | **Plasma gastrointestinal peptides in 48 h-fasted and 2 h-refed 160-day-old control and low-protein (LP) rats (n = 8 in each subgroup)**. Data are plotted as min to max values in box and whisker with $P < 0.05$ considered as significant difference between the fasted and refed subgroups (Mann–Whitney test).

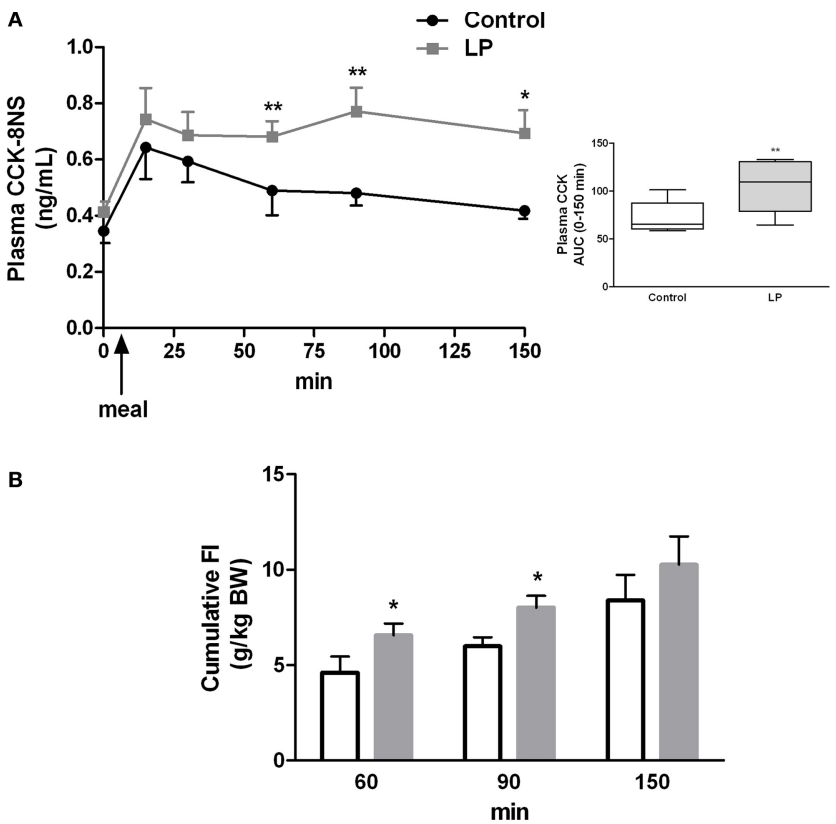

FIGURE 3 | **(A)** Basal and postprandial cholecystokinin (CCK) secretion measured in plasma at different times before and after refeeding by ELISA in control (n = 9) and low-protein (LP) rats (n = 9). In the box are represented the area under curve (AUC) calculated over the 0- to 150-min period. Rats were fasted 15 h before refeeding at light off indicated by an arrow. **(B)** Cumulative food intake [g/kg bodyweight (BW)] measured by food weighing in the same groups of rats. BWs (mean ± SD) were 658 ± 57 and 621 ± 90 g in control and LP groups, respectively. Values are means ± SEM. Significant differences between control and LP groups at $^*P < 0.05$ and $^{**}P < 0.01$, respectively (Mann–Whitney test).

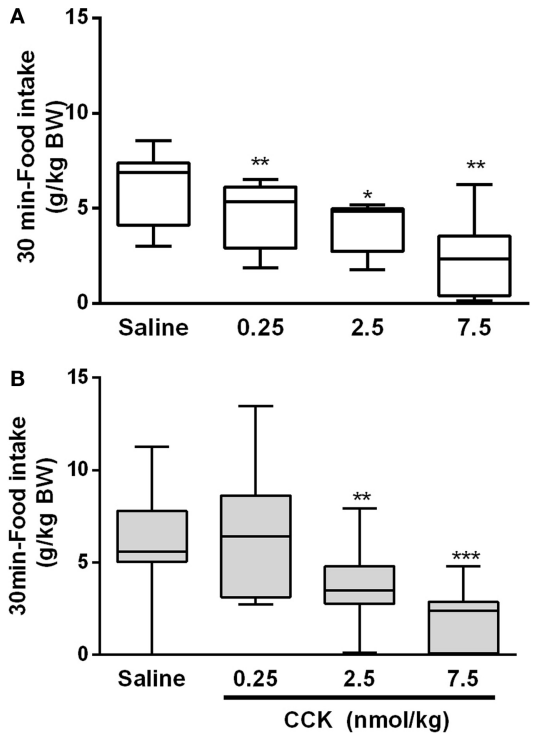

FIGURE 4 | Food intake during the first 30 min following i.p. injection of CCK and refeeding in control (A) and low-protein (LP) (B) groups. Bodyweights (mean ± SD) were 655 ± 58 and 590 ± 81 g in control and LP groups, respectively. Each rat received all treatments at different days (repeated measures) with a minimum of 3-day wash out (recovery) between two treatments. *$P < 0.05$, **$P < 0.01$ indicated significant differences vs saline group (Friedman and Wilcoxon signed rank tests).

Concomitant to higher CCK concentration at 60 and 90 min post-feeding in the LP group, cumulative FI measured for the same period was significantly higher in LP rats than control rats (**Figure 3B**). This result demonstrated that higher amount of plasma CCK was inefficient to reduce FI in LP rats.

Sensitivity to CCK-Induced Satiety

Since CCK is a major intestinal peptide of satiation, we examined its satietogenic effect by measuring FI after an i.p. administration of CCK-8S. As expected, in the control group, CCK induced a dose-dependent decrease of food ingestion in accordance with the normal sensitivity to satietogenic effect of CCK (**Figure 4A**). The minimal dose of CCK [0.25 nmol/kg; (28)] induced a 25% FI reduction ($P < 0.01$) on the first 30 min as compared to saline in the control group. In LP rats, this minimal dose had no effect on FI. A 10-fold higher dose (2.5 nmol/kg) was necessary to induce a significant reduction of FI in LP rats ($P < 0.01$). This suggested a resistance to the satiety effect of CCK (**Figure 4B**). At 60 and 90 min post-CCK injection and refeeding, FI was no more significantly different from saline in the both group except for the higher dose of CCK at 60 min ($P < 0.05$) (data not shown).

Vagal Neurochemical Phenotype and CCK-1R Signaling

Since short-term regulation of FI by CCK acts primarily *via* vagal nerve, the expression of CCK-1R was measured in nodose ganglia. The basal expression of vagal CCK-1R measured by immunofluorescence ($P < 0.01$) was lower in LP rats as compared to control rats and could contribute to the loss of sensitivity to CCK-induced satiety in LP rats (**Figures 5A,B**). No effect of

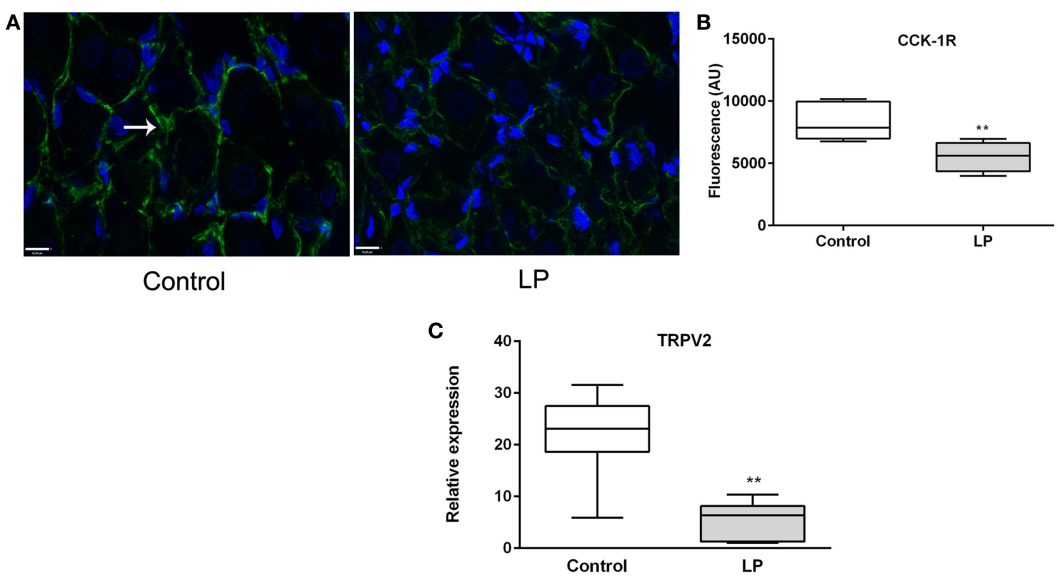

FIGURE 5 | (A) Immunofluorescence (×63) of CCK-1R (green) in nodose ganglia of control (left) and low-protein (LP) (right) rats at basal state (fasting). Nuclei are stained in blue with Dapi. **(B)** Corresponding histogram of quantification of fluorescence ($n = 3$–4). **(C)** Relative expression of TRPV2 expression at basal state. Values are means ± SEM. *$P < 0.05$, **$P < 0.01$ vs control (Mann–Whitney test).

refeeding on vagal CCK-1R expression was observed (data not shown) in the present experiment as previously reported (32). The neurochemical switch operated by CCK on VAN determines an anorexigenic vs orexigenic phenotype that mediates an appropriate response at the brain stem and hypothalamic levels to regulate FI (14, 15). Anorexigenic peptides (CART) and receptors (Y2-R) as well as orexigenic NPY were analyzed in nodose ganglia in response to refeeding in both groups. Unexpectedly, no significant difference was observed in expression of these genes between fasting and refed states, either in control or LP rats (Figure S2 in Supplementary Material).

Since CCK effects on VAN are mediated by TRPV2–5 cation channels (22), we determined gene expression of TRPV2 and found it significantly under expressed in LP nodose ganglia as compared to control (**Figure 5C**). Detection of phosphorylated CaMKII has been previously used as a marker of cellular activation in the nodose ganglia (23). Under basal conditions, the ratio pCaMKII/CaMKII was significantly lower in LP nodose ganglia as compared to control (0.4038 ± 0.06 vs 2.888 ± 0.47, respectively, means ± SEM, $P < 0.05$, Mann–Whitney test). Unexpectedly CCK-dependent pCaMKII/CaMKII ratio was reduced 20 min after injection in control rats, with no effect in LP rats (**Figure 6**).

Cholecystokinin activity on CREB phosphorylation in VAN (26) and phosphorylation of ERK in NTS is linked to the satiation effect of the peptide (21, 33). However, 20 min after exogenous administration of CCK, no activation of the ERK pathway was detected in nodose ganglia of control or LP rats (**Figure 7**).

DISCUSSION

Although some experimental studies testify alteration of FI and meal pattern in LP rats at adulthood, to the best of our knowledge the present data provide the first evidence that the sensitivity to CCK-induced satiation is impaired by perinatal undernutrition. The resistance to the satiation effect of CCK could be related to a lower expression of CCK-1R and TRPV2 in nodose ganglia in LP rats accredited by a lower basal and phosphorylated level of CaMKII as compared to control rats. The higher postprandial CCK release that we observed in LP rats possibly represents an adaptive mechanism that is partially inefficient at reducing the first meal after a fasting period.

Short-term FI and Postprandial CCK Are Altered in LP Rats

Alteration of short-term FI has been previously reported in perinatally malnourished rats. Protein restricted diet provided to dams during gestation and/or lactation modify the early appetite of their pups. They demonstrate a hyperphagic phase especially after weaning (7, 34) and during their catch-up growth (6, 7). At adulthood, FI seems grossly normal but a delay in satiation (7) and an increase of the first-meal size following 48-h fasting are still observed suggesting a persistent alteration of short-term regulation of FI. Such an alteration could predispose to obesity particularly when the animals are challenged with a high-calories diet (8). Interestingly, a very recent study showed that LP rats are hyperphagic at older age

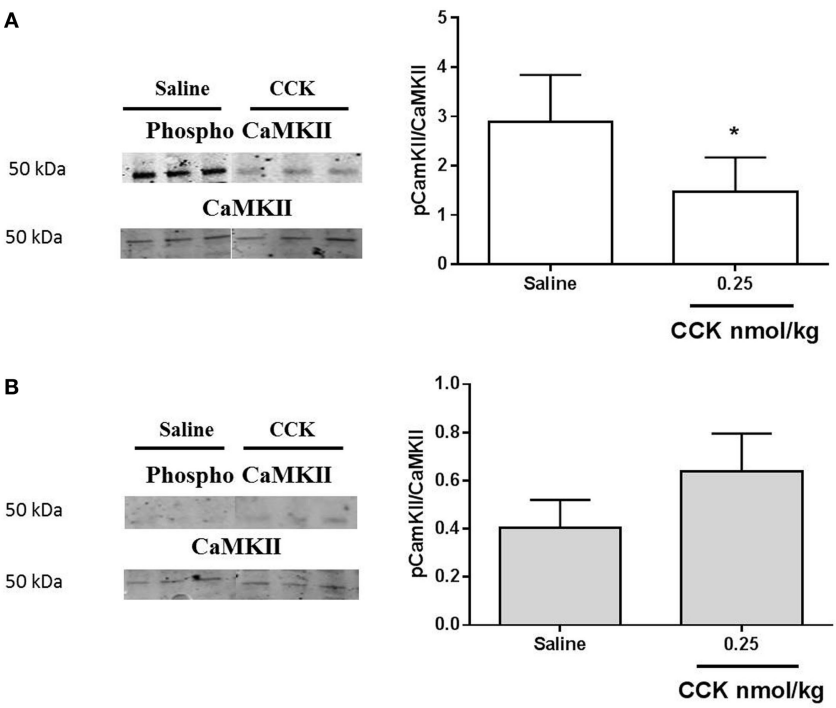

FIGURE 6 | Western blot analysis of phosphorylated and total calcium/calmodulin-dependent protein kinase (CaMKII) in nodose ganglia of (A) control and (B) low-protein rats in response to i.p. saline or CCK (0.25 nmol/kg bodyweight). Values are expressed as the ratio of phosphorylated protein/total protein. *$P < 0.05$: saline vs CCK (Mann–Whitney test).

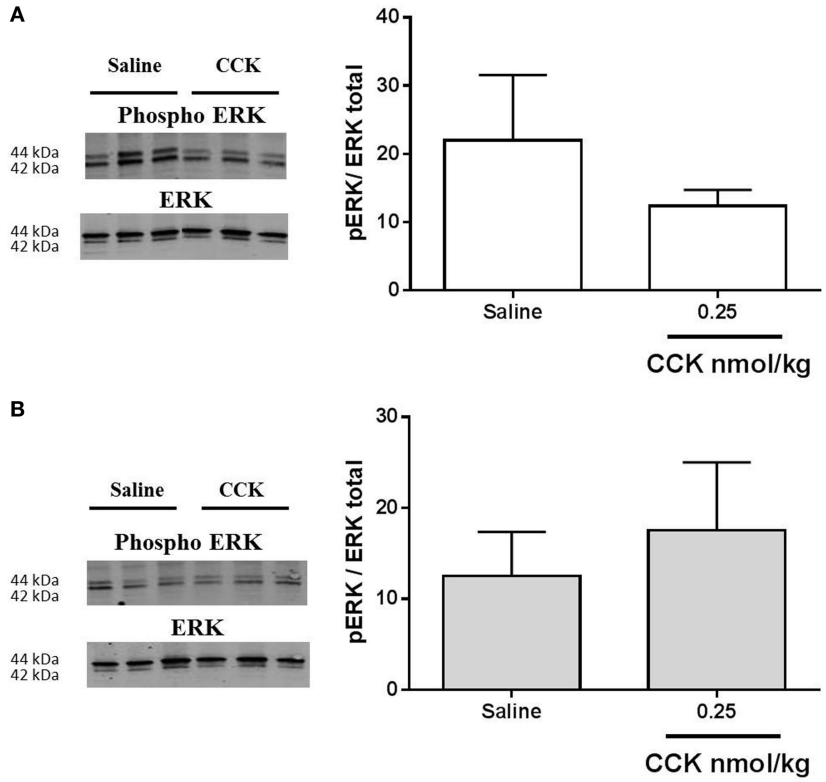

FIGURE 7 | Western blot analysis of phosphorylated and total ERK1/2 in nodose ganglia of (A) control and (B) low-protein rats (n = 4–5) in response to i.p. saline or CCK (0.25 nmol/kg bodyweight). Values are expressed as the ratio of phosphorylated protein/total protein. *P < 0.05: saline vs CCK (Mann–Whitney test).

(1-year old) than younger adult ages usually studied, reinforcing the importance of long lasting impact of programming hyperphagia by perinatal nutrition (35). In the present study using physiological cages that give precise information of meal pattern, we confirmed that adult (160-day old) LP rats present an altered regulation of the first-meal pattern after a long period of fast.

Short-term FI is regulated by a vago-vagal reflex initiated by arrival of food in the stomach and the upper intestine that are sensitive to both distension and nutrients. It ensues digestive secretion and release of appetite-regulating gut peptides (CCK, serotonin, GIP, PYY, glucagon like peptide-1, etc.), all known, at the exception of ghrelin, to reduce meal and delay the next meal (36). We first hypothesized that gene expression as well as the basal and postprandial release of those peptides may be altered in LP rats. A previous study reported elevated plasma ghrelin and reduced CCK and PYY in adult IUGR rats obtained by a 50%-caloric restriction of their dams diet during gestation and maintained restricted during lactation (37). These changes were paralleled to mRNA levels in gastric and intestinal tissues. In this drastic model, as previously shown by others (38), rats are hyperphagic during the whole period of the experiment (from weaning to 7- to 9-month olds). However, only fasting GI peptides concentrations were reported in that study. Therefore, no conclusions on their dynamic change in relation to FI can be drawn. In the present work, offspring obtained by protein restriction of theirs dams during gestation and lactation showed no modification in gene expression of GI peptides, nor at basal or postprandial states. We then measured their plasma concentrations and found that only postprandial CCK was significantly higher than basal in 2-h-refed LP animals. This result seems in contrast with the increased first-meal size and the reduced satiety ratio we measured in LP rats but can be interpreted as if a higher CCK release was inefficient to correctly regulate FI. The kinetic of plasma CCK release post-refeeding was similar in both groups suggesting no delay in CCK secretion by I-cells. By contrast, the feedback regulation seen by the reduced FI 1 h post-refeeding in control rats seemed ineffective in LP rats. As previously mentioned, short-term regulation of FI is initiated by proximal gut mechano- and chemoreceptors, in association with GI peptides release. These initials signals are integrated by VAN to the hindbrain, which triggers a vago-vagal reflex to decrease FI by inhibiting gastric emptying, stimulating digestive secretion, etc. The release of GI peptides is supposed to be regulated by a classic downregulation of theirs receptors once stimuli (nutrients) have moved to distal parts of gut. Thus, the higher postprandial plasma CCK found in LP rats led us to propose the existence of a state of resistance to the satietogenic effect of CCK in these rats.

LP Rats Are Resistant to CCK-Induced Satiation

Exogenous administration of CCK-8S in LP rats at a dose previously shown to induce satiety in refed rats (28) was not efficient to reduce FI in contrast to control group where the consumption of food was 25% reduced. Since the satietogenic effect of CCK is mediated by CCK-1R on VAN, we hypothesized that perinatal malnutrition could affect CCK signaling *via* its receptor on the vagus nerve. We effectively found that CCK-1R immunoreactivity was significantly lower in nodose ganglia of LP rats compared to control rats probably contributing to the resistance to CCK. A reduced sensitivity to a satietogenic dose of CCK, leading to hyperphagia, has already been shown in obesity-prone rats receiving standard chow (39) as well as in high-fat diet fed rats (40). In diet-induced obese rats, neurochemical analysis of VAN supports a vagal resistance to CCK and leptin in this model in which hyperphagia occurred concomitantly with this resistance (29). In DIO mice, spontaneous activity of VAN innervating the jejunum is weaker as compared to control and the number of afferent neurons that respond to CCK is reduced (41). The impact of perinatal malnutrition on the activity/neurochemical phenotype of the vagus nerve is poorly documented. One study reported a reduced vagal firing rate in adult rats reared by mothers fed a LP diet leading to an impaired efferent vagal activity (42). Electrophysiological studies of nodose neurons of LP rats are in progress in our lab to better characterize the effect of perinatal malnutrition on the activity of the vagus nerve.

Dockray and collaborators have considered CCK as a gatekeeper of the vagal phenotype, switching from an orexigenic phenotype during fasting at low CCK concentration to an anorexigenic one when CCK is released at refeeding (16, 25, 43). Here, we did not observe any modifications of the vagal phenotype between fasting and refed states neither in control nor in LP rats. This discrepancy could be related to different duration of fasting between experiments, even if our conditions (48-h fasting) were close to that of the earlier studies (24–48 h). A very recent study in mice also failed to reproduce this metabolic switch of VAN from an anorectic to orexigenic phenotype (44). Using confocal microscopy to visualize all afferent visceral C-fibers of the vagus nerve, these authors showed that the neuropeptide CART was not regulated by metabolic challenge (fasting or high-fat diet). They also failed to detect any production of MCH, an orexigenic neuropeptide previously shown to be produced by CART neurons in fed state in response to fasting. In our study, we did not detect MCH (mRNA or protein) in nodose ganglia of control and LP rats, whatever they were in their fed or fasted state (data not shown). Thus, our data could not support the hypothesis of an alteration of the vagal phenotype switch by CCK in LP rats but led us to consider CCK signaling in nodose ganglia.

Following its receptor activation, in synergic interaction with leptin receptors, CCK induces a cascade of signal transduction pathways leading to neuronal firing (45). Among them, p-ERK1/2 in terminal endings of VAN in NTS is central to CCK-induced inhibition of FI (19, 21). In the present study, we did not measure any activation of ERK following CCK injection in nodose ganglia. Previous data showing CCK-induced phosphorylation of ERK in NTS reported that the effect occurred very quickly, as soon as 6 min following injection (21). Here, we measured CCK signaling 20 min after stimulation, this may have contributed, in combination with the low dose used, to the absence of phosphorylation signal in nodose ganglia. An analysis on a shorter time needs to be further performed and CCK signaling measured at the afferent endings in the NTS may provide a better comprehension of the underlying mechanism. Concerning calcium signaling, we showed that LP lowered TRPV2 expression in nodose ganglia. This observation could be put in relation with the attenuated level of CaMKII and pCaMKII measured in this model as compared to normal birth-weight rats. Such a reduced expression of CaMKII has been previously reported in the frontal cortex in young adult LP rats (46). Similarly, in LP fetal brain, number of CaMKII immunopositive cells was decreased as compared to control (47). The major role of CaMKII in mediating glutamate signaling has been extensively studied in postsynaptic events implied in memorization and cognition, which are altered in IUGR infants and animal models (48). As proposed by Flores et al., underexpression of CaMKII in frontal cortex of LP rats could be related to altered synaptic plasticity and decreased learning performances in this model. In our study, the reduced expression of CaMKII in nodose ganglia may contribute, together with the reduced CCK-1R expression, to the hyposensitivity of afferent neurons to CCK. Actually, factors leading to this decreased basal expression related to perinatal LP environment are unknown. Epigenetic mechanism is now widely accepted as a memory of antenatal undernutrition exposure throughout life. Research of epigenetic marks on the promoter of the CaMKII gene would be of great interest to link early LP environment and CaMKII expression. More unexpectedly, exogenous CCK induced a decrease in phosphorylation of CaMKII in nodose ganglia of the control group of rats. It has been previously demonstrated in cultured nodose neurons that the CCK-induced increase in cytosolic calcium concentrations is dependent on extracellular calcium influx rather than mobilization on intracellular stores (49). In neurons of the dorsal root ganglia, such a decrease in CaMKII autophosphorylation has been reported *in vitro* by depleting extracellular calcium (50). The significance of this observation in the present study needs further investigation.

In conclusion, we showed for the first time in the present study that adult perinatally undernourished rats have a reduced first-meal satiety ratio associated to a higher postprandial plasmatic CCK release, a reduced sensitivity to CCK when injected at low concentration and a reduced presence of CCK-1R and TRPV2 in nodose ganglia. Altogether, the present data demonstrated a reduced vagal sensitivity to CCK in LP rats at adulthood, which could contribute to deregulation of FI reported in this model.

ETHICS STATEMENT

All experiments were conducted in accordance with the European Union regulations for the care and use of animals for experimental procedures (2010/63/EU). Protocols were approved by the local Committee on the Ethics in Animal Experiments of Pays de la Loire (France) and the French Ministry of Research (Projects

2011.4 and 0271.01). Animal facility is registered by the French Veterinary Department as A44276.

AUTHOR CONTRIBUTIONS

GLD designed the study, GLD and CP wrote the protocol, and MN wrote the first draft of the manuscript. MN and CP managed the literature searches and performed animal experiments and analyses. MN performed the statistical analyses. PP contributed for interpretation of data and revised the manuscript. All authors contributed to and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2017.00065/full#supplementary-material.

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Annexe 3. Publication #4.

nutrients

MDPI

Article

Neonatal Consumption of Oligosaccharides Greatly Increases L-Cell Density without Significant Consequence for Adult Eating Behavior

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Abstract: Oligosaccharides (OS) are commonly added to infant formulas, however, their physiological impact, particularly on adult health programming, is poorly described. In adult animals, OS modify microbiota and stimulate colonic fermentation and enteroendocrine cell (EEC) activity. Since neonatal changes in microbiota and/or EEC density could be long-lasting and EEC-derived peptides do regulate short-term food intake, we hypothesized that neonatal OS consumption could modulate early EECs, with possible consequences for adult eating behavior. Suckling rats were supplemented with fructo-oligosaccharides (FOS), beta-galacto-oligosaccharides/inulin (GOS/In) mix, alpha-galacto-oligosaccharides (αGOS) at 3.2 g/kg, or a control solution (CTL) between postnatal day (PND) 5 and 14/15. Pups were either sacrificed at PND14/15 or weaned at PND21 onto standard chow. The effects on both microbiota and EEC were characterized at PND14/15, and eating behavior at adulthood. Very early OS supplementation drastically impacted the intestinal environment, endocrine lineage proliferation/differentiation particularly in the ileum, and the density of GLP-1 cells and production of satiety-related peptides (GLP-1 and PYY) in the neonatal period. However, it failed to induce any significant lasting changes on intestinal microbiota, enteropeptide secretion or eating behavior later in life. Overall, the results did not demonstrate any OS programming effect on satiety peptides secreted by L-cells or on food consumption, an observation which is a reassuring outlook from a human perspective.

Keywords: prebiotic; gut-brain; programming; microbiota; L-cell; eating behavior

1. Introduction

Preventing unhealthy feeding behavior is highly desirable since deleterious eating habits are associated with health problems, including a higher risk of overweight and obesity [1]. Since eating behavior is the result of integrated central and peripheral biological systems that are influenced by genetic, psychological, and environmental factors [2], its optimization is highly complex and requires the full elucidation of the mechanisms that control eating behavior. Central regulation of appetite is mediated by peripheral inputs generated by stomach distension, through signals from the gut epithelium when it senses the availability of nutrients, such as satiety-regulating peptides synthetized and released by enteroendocrine cells (EECs), as well as by long-term energy signals released by adipose tissue and cerebral inputs generated by hedonics and rewards circuits [2,3].

In addition to the evident progress in understanding these interconnections, recent advances include two major findings: first, eating behavior may be programmed very early in life, and second, it could be regulated by intestinal microbiota.

According to the developmental origin of health and disease (DOHaD) theory, adverse early-life conditions may predispose a person to disordered eating [4]. Among the environmental stressors that may have an effect, it is suggested in both animal and human studies that perinatal nutrition could program the appetite (see [5,6] for reviews). In rodents, experiments based on restricting maternal nutrition and/or manipulating litter size have demonstrated that both pre- and post-natal nutrition may alter food intake [7–9] and/or food preference [10] in offspring, with subsequent repercussions in adulthood. In humans, although controversial results have been observed concerning the influence of prenatal nutrition on later eating behavior (see [11] for review), some observational evidence suggests that early nutrition/growth affects appetite regulation [12–14] and food preference programming, as demonstrated after repeated exposure to new flavors [15].

With regard to the involvement of intestinal microbiota in feeding behavior, although it has been known for several years that fermentation catalyzed by intestinal microbiota stimulates the expression of satiety peptides by EECs [16,17], it is only recently, in connection with the growing appreciation of the role that intestinal microbiota play in regulating host physiology, that this topic has generated renewed interest [18,19]. As pointed out in these reviews, some observations objectively support the involvement of intestinal microbiota in the regulation of feeding behavior. Thus, in ascending order of convincing power, we can quote: (i) the differences observed in microbiota composition or diversity in patients with anorexia nervosa (see [18] for review, [20]); (ii) the fact that feeding behavior differs between germ-free and conventional animals (see [21] for an example), (iii) the ability of certain microbiota modulating agents—e.g., certain prebiotic oligosaccharides [22,23]—to affect feeding behavior, and (iv) the delineation of mechanistic pathways that link microbiota with central and peripheral neuroendocrine systems responsible for feeding behavior, a finding which supports the existence of a causative link. For example, EECs that secrete appetite-regulating peptides can be mentioned since they have a large diversity of receptors enabling them to sense microbial inputs such as fermentation-derived short chain fatty acids (SCFA), secondary biliary salts or pathogen-associated molecular patterns [see 18 for review].

Reconciliating these two emerging issues related to the regulation of feeding behavior, i.e., its possible programming in early life and its control by intestinal microbiota, we hypothesized that early modifications to microbiota may program adult feeding behavior. This programming could stem from either the programming of intestinal microbiota (e.g., [24]) or the early impacts of microbiotal changes with long-lasting consequences for the peripheral neuroendocrine systems that control adult feeding behavior and/or the central sensing of it. In this respect, it is worth mentioning the ability of microbiota-modulating agents to affect the hypothalamic expression of neurogenic factor (BDNF) during the neonatal stage [25], and the potential programmable character of both the EECs [26] and the vagal sensitivity [9]. In addition, the putative ability of the gut microbiota to act through epigenetic mechanisms (see [27] for review) as well as the ability of the microbiota presence [28] and certain microbiota modulating agents [e.g., in the case of prebiotics [29,30]) to modulate some behaviors in adults mice can be cited, assuming that they are transposable in the neonatal period.

Using the rat as a model, we therefore evaluated whether neonatal modulation of the microbiota induced by prebiotics could program eating behavior and the secretion of gastrointestinal peptides in adulthood. We first verified that the presuppositions underlying our hypothesis were present in our case, by investigating the immediate impact of the neonatal prebiotic supplementation on both the intestinal microbiota and the maturation and functioning of EEC in suckled rats. We decided to use indigestible oligosaccharides (OS) to modify the intestinal microbiota of the neonatal rats for two reasons: first, OS are recognized as intensively-fermented prebiotics [31], which are also operant in neonatal rats [24] and infants (see [32] for review) and have been shown to stimulate EEC proliferation

and activity in adult animals [33,34]; second, they represent relevant nutrients in neonatal nutrition since they are commonly added to infant formula to better mimic maternal milk [35].

2. Materials and Methods

2.1. Ethics Statement

All experiments were conducted in accordance with the European Union Directive on the protection of animals used for scientific purposes (2010/63/EU). The protocols were approved by the Ethics Committee for Animal Experiments for the Pays de la Loire region (France) and the French Ministry of Research (APAFIS#3652-20 160 1 1910192893 v3). The animal facility is registered by the French Veterinary Department as A44276.

2.2. Animal Experiment

Primiparous female Sprague-Dawley rats (n = 16) were obtained on day one of gestation (G1) from Janvier-labs (le Genest Saint Isle, France) and housed individually (22 ± 2 °C, 12:12-h light/dark cycle) with free access to water and chow (A03, Safe Diet, Augy, France). At birth, 8 litters were culled to 8 male pups per mother with systematic cross fostering as previously described [24]. From day 5 to day 14/15 of life (PND5 to PND14/15), the pups were given various solutions of FOS, GOS/In mix (9:1), αGOS or a mix of the monomers present in the OS solutions (Table 1) by oral gavage. These OS were selected either because they are already used in infant formula (GOS/In, FOS [35]) or because they constitute a new source of OS, the physiological properties of which are to be characterized (αGOS). Two pups from each litter were given one of the 4 solutions daily.

Table 1. Composition of solutions administered by gavage to pups from PND5 to PND14/15 ($g \cdot mL^{-1}$).

	CTL	FOS	GOS/In	αGOS
GOS syrup (VivinalGOS, FrieslandCampina Domo, LE Amersfoort, The Netherlands)			0.65	
Inulin powder (Raftiline HP, BENEO-Orafti S.A., Tienen, Belgium)			0.03	
FOS powder (Beneo P95, BENEO-Orafti S.A., Tienen, Belgium)		0.34		
αGOS powder (Olygose, Venette, France)				0.32
α-Lactose monohydrate (L3625, Sigma-Aldrich, St. Quentin Fallavier, France)	0.096	0.096		0.096
D(+)-glucose monohydrate (108342, Merck Santé SAS, Fontenay sous Bois, France)	0.087	0.082		0.087
D(+)-galactose monohydrate (104058, Merck Santé SAS, Fontenay sous Bois, France)	0.005	0.005		0.004
D(−)-fructose (F0127, Sigma-Aldrich, St. Quentin Fallavier, France)	0.015		0.015	0.015
Saccharose (S9378, Sigma-Aldrich, St. Quentin Fallavier, France)	0.002		0.002	0.002
Total oligosaccharides [§]		0.30	0.30	0.30
Total digestible sugars [§ !!]	0.20	0.20	0.20	0.20

CTL, control; FOS, fructo-oligosaccharides; 93.2% dry matter composed of 90.4% oligomers and 6.6% monomers, providing 0.015 $g \cdot mL^{-1}$ of fructose, 0.005 $g \cdot mL^{-1}$ of glucose and 0.002 $g \cdot mL^{-1}$ of saccharose; GOS/In, mix (9:1) of galacto-oligosaccharides and long chain fructo-oligosaccharides (In, inuline). For GOS: 75% dry matter composed of 59% oligomers and 41% monomers; for inulin: 97% dry matter composed of 99.5% oligomers, the mix was providing 0.095 $g \cdot mL^{-1}$ of lactose, 0.086 $g \cdot mL^{-1}$ of glucose and 0.005 $g \cdot mL^{-1}$ of galactose; αGOS: alpha galacto-oligosaccharides (95.9% dry matter composed of 99.4% oligomers, providing 0.001 $g \cdot mL^{-1}$ of galactose. [!!, §] These calculations take into account the dry matter of the components, their purity, and the amount of digestible sugars they contain.

The pups were weighed daily and the administered volume was adapted to body weight to reach 3.2 g/kg in order to approximate the dosage actually consumed by babies fed with prebiotic enriched formula, taking into account both the difference in metabolic rate between rats and humans and the true prebiotic content of infant formula [35].

Four of the 8 litters were used for our main objective, i.e., to assess eating behavior programming: rats from these 4 litters (n = 8 per group) were weaned at PND21 onto standard chow (A03, Safe Diet, Augy, France) in individual cages until PND124/126, when they were sacrificed by decapitation after induction of deep anesthesia (isoflurane/O_2, 5 $L \cdot min^{-1}$). During the follow-up, food consumption was measured 3 times a week. Rats from the 4 remaining litters (n = 8 per group except for FOS where

n = 7 as explained below) were sacrificed at PND14/15 by the method described above to investigate the immediate impact of the neonatal prebiotic supplementation on both intestinal microbiota and the maturation and functioning of EECs.

This experimental set-up was designed to form 8 supplemented males, originating from 4 different litters, per group at each studied age. Due to the death of one of the pups during the supplementation period (this pup was then replaced by an untreated one to equilibrate the litter size), the number of pups in the FOS group at PND14/15 was reduced to 7. These values are maximum numbers that are not always found in each of the analyses (see the illustration legends). This stemed from either physiological reasons (e.g., 2 animals did not eat at all during the fasting-refeeding test), or because of quality requirements (e.g., reliable data from in physiological cages could only be obtained for n = 7 in CTL and GOS/In groups; n = 6 in FOS group and n = 5 in αGOS group), or statistical inconsistency (e.g., outliers identified by the Dixon's Q test were excluded in RT-qPCR analysis as well as food/beverage consumption follow-ups), or technical problems (e.g., accidental spillage of supernatant before analysis of bacterial end-products or sequencing failure during 16S rDNA analysis or poor quality of some tissue sections in the case of immunochemical analysis). Nevertheless, in all analyses, the 4 different litters were always represented.

2.3. Tissue Collection

Under anesthesia, intracardiac blood was collected in a tube containing EDTA (Microtubes 1.3 mL K3E, Sarstedt MG & Co, Marnay, France) and plasma collected after centrifugation 2000× g, 15 min, 4 °C) was frozen at −20 °C for further analysis. The contents of the most distal 15 cm of the ileum were harvested by flushing, using 1 mL of Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific, St-Herblain, France), and the cecocolonic (PND14/15) or cecal (PND124/126) content was collected, weighed, mixed with 5-fold or 2-fold their volume of sterile water (PND14/15 and PND124/126, respectively). After complete homogenization, these cecocolonic/cecal suspensions were centrifuged 7800× g, 20 min, 4 °C) then both supernatants and pellets were frozen at −20 °C for analysis of the fermentation end-products (SCFA and lactate) and microbiota, respectively. Intestinal tissues (ileum and proximal colon) were rapidly collected and frozen in liquid nitrogen for RNA analysis. Additional tissue samples were fixed in 4% paraformaldehyde for immunofluorescence analysis.

2.4. Eating Behavior

2.4.1. Meal Pattern

Between PND74 and PND99, eating behavior was analyzed in physiological cages (Phecomb cages, Bioseb, Vitrol, France) as previously described [8]. Briefly, the rats were housed individually and following 24 h of acclimatization to the cage and refilling with fresh food between 9.00 a.m. and 11.00 a.m., data were recorded every 5 s over a 20-h period. Due to the intervention during the diurnal phase, the analysis was reduced to 8 h whereas the nocturnal phase was 12 h. The exact feeding pattern was defined with a minimal size of 0.1 g, a minimum duration of 10 s and a minimum inter-meal interval of 10 min. Events such as large vibrations (contact with the feed tray without eating) were filtered by the Phecomb system monitoring software (Compulse v1.1.01). The reliability percentage of the quality signal was calculated by the software and only experiments with a percentage >80% were used. Meal parameters extracted from Compulse software included number of meals, meal size and duration, inter-meal intervals and satiety ratio.

2.4.2. Taste Preference

Preference for sweet taste was measured at PND110 using the bottle test experiment [36]. After a two-day habituation to the presence of two bottles in their own cages, the animals had the choice of the two bottles, one containing tap water and the other 0.05% saccharin (Sigma-Aldrich, St. Quentin Fallavier, France). Drink intake was measured daily for three days. The position of the two bottles was

reversed each day to prevent position preference bias. The sweet preference score was calculated as the ratio between the volume of saccharin solution consumed and the total drink intake in 24 h, then multiplied by 100. Preference was defined as a percentage greater than 50.

2.5. Fasting-Refeeding Test, Kinetics of GLP-1 and PPY Release and Response to Glucose

At PND105, a 4 h kinetic of GLP-1 and PYY release in plasma was carried out. Rats were not fed for 16 h to induce hunger and then fed for 20 min with a calibrated quantity of chow (A03, safe Diet). Food intake was weighed at the end of the 20 min period. Any crumbs that fell in the cage were weighed and deducted from the food intake. Blood samples were collected from the tail vein in tubes containing EDTA (Microvette CB300 EDTA 3K, Sarstedt, Marnay, France) at 0 (15 min before refeeding), 30, 60, 120, and 180 min after the beginning of the meal.

At PND124/126, the rats were not fed for 16 h, and 2 h before being sacrificed they were given an oral bolus of glucose (2 kg/kg BW) in order to challenge glucose sensing in GLP-1/PYY-producing EECs.

2.6. Plasma Gastrointestinal Peptides

The plasma concentration of total GLP-1 and total PYY was assayed by the ELISA technique using kits from Millipore (Merck- Millipore, Molsheim, France) and Phoenix Pharmaceutical (Phoenix France S.A.S, Strasbourg, France), respectively.

2.7. Fermentation End-Products

Ileal and cecal supernatants were centrifuged 8000× *g*, 20 min, 4 °C, diluted (1/10) with 0.5 M oxalic acid and SCFA (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) were analyzed by gas chromatography as previously described [37]. The D-and L-lactates were measured in the supernatants after heating to 80 °C for 20 min with a D/L-lactic acid enzymatic kit following the manufacturer's instructions (Biosentec, Toulouse, France).

2.8. Immunochemistry

Tissue sections (4–5 µm) of fixed ileum and proximal colon were double-stained with a goat polyclonal antibody raised against GLP-1 diluted at 1/200 (Santa Cruz Biotechnology Inc, Santa Cruz, USA) and a rabbit anti-chromograninA (chrgA, diluted at 1/1000 (ImmunoStar Inc, Hudson, USA), followed by incubation with anti-goat and anti-rabbit fluorescent secondary antibodies (1/1000). Nuclei were counterstained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, St. Quentin Fallavier, France). Tissues sections were mounted in Prolong Gold anti-fading medium (Molecular Probes, Thermo Scientific, Courtaboeuf, France). Three sections per sample were analyzed with a Nanozoomer (×20) (Hamamatsu Photonics France, Massy, France). The number of fluorescent cells along the crypt-villus axis unit was counted twice by a blind operator, using the NDP view software (Hamamatsu, Photonics France, Massy, France). A total of 40 to 60 crypt-villus units per section were counted.

2.9. Quantitative Real-Time PCR

Total RNA extraction from the ileum and colon was carried out using a QIAamp RNA Blood Mini kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. Two micrograms of RNA were reverse-transcribed using M-MLV reverse transcriptase (Promega, Charbonnières-les-Bains, France). Five microliters of 1/40 dilution of cDNA solution were subjected to RT-qPCR in a Bio-Rad iCycler iQ system (Biorad, Marnes-la-Coquette, France) using a qPCR SYBR Green Eurobiogreen®Mix (Eurobio, Les Ulis, France). The quantitative PCR consisted of 40 cycles, 15 s at 95 °C, 15 s at 60 °C and 15 s at 72 °C each. Primers sequences are shown in Table S1 of the Supplementary Material. For quantification of Neurog3, rat PrimePCR™ SYBR®GreenAssay Neurog3 (Biorad,

Marnes-la-Coquette, France) was used. Relative mRNA quantification was expressed using the $2^{-\Delta\Delta Cq}$ method with actin gene as a reference.

2.10. Bacterial 16S rDNA Sequencing of Cecal Contents

DNA was extracted from pellets of ceco-colonic content (max 250 mg) using the QIAamp Fast DNA Stool Mini kit (Qiagen, Courtaboeuf, France) after enzymatic and mechanical disruptions as described previously [37] except that homogenization was carried out at 7800 rpm for 3 × 20 s intervals with 20 s rest between each interval in a Precellys® "evolution" bead-beater (Bertin, Montigny-le-Bretonneux France). The V4 hyper-variable region of the 16S rDNA gene was amplified from the DNA extracts during the first PCR step using composite primers (5'-CTTTCCCTACACGACGCTCTTCCGATCTGTGY CAGCMGCCGCGGTAA-3' and 5'-GGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGG TWTCTAAT-3') based on the primers adapted from Caporaso et al. (i.e., 515F and 806R) [38]. Amplicons were purified using a PP201 PCR Purification Kit (Jena Bioscience, Jena, Germany). Paired-end sequencing was performed on a HiSeq 2500 System (Illumina, San Diego, CA, USA) with v3 reagents, producing 250 bp reads per end, following the manufacturer's instructions by the GeT+-PlaGe platform (INRA, Toulouse, France). The 16S rDNA raw sequences were analyzed with FROGS v2 pipeline (http://frogs.toulouse.inra.fr/) [39]. After de-multiplexing, quality filtering and chimera removing, the taxonomic assignments were conducted for OTUs with abundance >0.005% with Blast using Silva 128 database containing sequences with a pintail score at 80 to determine the bacterial compositions. FROGSSTAT Phyloseq tools were used to normalize raw abundances by rarefaction and to calculate alpha and beta diversity indices.

2.11. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, USA) or R (librairies "stats v3.5.1" and "corrplot v0.84", [40]). Differences between treatments were searched using one-way ANOVA followed by Tukey's multiple comparison tests for most data, with the exception of growth and food consumption data which were subjected to multiple t-tests with correction for multiple comparison using the Holm-Sidak method. Sweet taste preference test was analyzed by the one sample t-test to compare to compare data against the 50% (no preference) value. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Neonatal OS Supplementation Did Not Substantially Affect Rat Growth

Both FOS and αGOS supplementation was associated with a significant transitory reduction of pup growth in the first days of intervention (PND7 to PND10 and PND6 to PND8 respectively, Figure S1). When compared with body weights from the CTL group, the differences observed were only 9.1 to 11.5% and did not significantly affect the cumulative weight gains measured either from birth until the end of supplementation or for the whole lactation period (Table 2).

Table 2. Bodyweight gain (g) during lactation.

Treatment	BW Gain PND0-14	BW Gain PND0-20
CTL	30.4 ± 4.2 [1]	50.5 ± 6.0
FOS	28.0 ± 3.4	45.7 ± 4.6
GOS/In	29.4 ± 3.3	49.6 ± 5.8
αGOS	27.9 ± 2.7	46.8 ± 5.1

[1] Data are means ± SD collected from the total effective of rats (n = 15–16 per group during PND0-14 and n = 8 during PND0-20). BW, bodyweight.

No significant differences in bodyweight were observed between groups after weaning (Figure S2).

3.2. Neonatal OS Supplementation Exerted a Marked Immediate Impact on Intestinal Environment

3.2.1. OS Supplementation Modified Both Composition and Activity of Neonatal Intestinal Microbiota

Following 16S rDNA sequencing, no significant differences were noticed in raw sequence numbers between cecocolonic samples collected at PND14/15 (355,245 ± 10,367, 30,306 ± 13,817, 40,275 ± 18,343 and 31,808 ± 10,101 for CTL, FOS, GOS/In and αGOS, respectively) or in percentages of sequences kept after quality filtering (83.8 ± 4.0, 76.4 ± 18.4, 83.9 ± 4.2, and 81.7 ± 7.1). The cecocolonic contents of animals supplemented with OS exhibited similar reductions in richness ($p < 0.001$) compared with CTL animals (Chao1 values: 66.2 ± 21.0, 72.9 ± 28.1, and 73.9 ± 35.3 for FOS, GOS/In and αGOS, respectively *versus* 180.0 ± 35.7 for CTL). The cluster dendrogram generated using weighed UniFrac metric which illustrates beta or between-sample diversity, highlighted an obvious dissimilarity between the microbiotas of the OS-supplemented animals and those of animals from the CTL group (Figure 1) but did not reveal any effect of the nature of the OS.

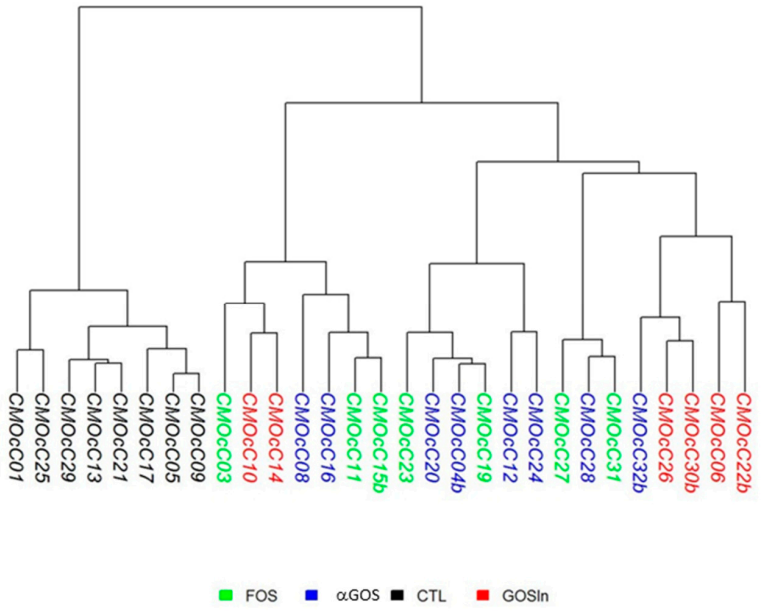

Figure 1. Hierarchical clustering based on the Ward's method of phylogenetically informed distance matrix computed using the weighted UniFrac metric for cecocolonic contents collected at postnatal day (PND) 14/15 (n = 6 to 8 per group).

When considering bacterial families occurring at more than 0.01% of the total sample abundances (Table 3), the OS impact was typified by significant decreases in Lactobacillaceae, Bacteroidales S24-7 group, Prevotellaceae, Streptococcaceae, Peptococcaceae, Coriobacteriaceae, Aerococcaceae, Family XIII, and Rikenellaceae. In addition, OS supplementation decreased Ruminococcaceae abundance but this impact only reached statistical significance for FOS and αGOS. These decreases in relative abundance were differently compensated according to the OS: increases in Bifidobacteriaceae reached statistical significance following FOS and αGOS supplementations, Enterobacteriaceae increased following αGOS supplementation and Lachnospiraceae increased following GOS/In supplementation.

Significant differences between OS were scarce and only occurred between GOS/In and αGOS in their impact on Lachnospiraceae (Table 3).

Concurring with these compositional changes, the 10-day supplementation greatly affected fermentation end-product concentrations in both ileal and colonic contents at PND14/15.

In the ileum, lactate concentration was below the detection limit (0.22 mM) in all animals, and the concentration of acetate—the sole SCFA present at this age in this intestinal segment—was significantly increased ($p < 0.005$) through FOS supplementation (6.9 ± 3.6 mM) compared to CTL (0.3 ± 0.4 mM), GOS/In (1.6 ± 2.0 mM) and αGOS (0.6 ± 0.8 mM).

Table 3. Relative abundances (%) for families with abundances > 0.01% at PND14/15 according to the postnatal OS supplementation.

Family	CTL	FOS	GOSIn	αGOS
Actinomycetaceae	0.095 ± 0.084 [1]	0.028 ± 0.032	0.076 ± 0.056	0.068 ± 0.058
Aerococcaceae	0.086 ± 0.029 [a,2]	0.011 ± 0.011 [b]	0.015 ± 0.012 [b]	0.020 ± 0.019 [b]
Alcaligenaceae	0.020 ± 0.041	0.030 ± 0.047	0.378 ± 0.576	0.036 ± 0.053
Bacteroidaceae	2.352 ± 0.991	6.510 ± 10.047	6.837 ± 5.729	3.719 ± 5.492
Bacteroidales.S24.7 group	6.812 ± 2.953 [a]	0.053 ± 0.080 [b]	0.098 ± 0.094 [b]	0.084 ± 0.103 [b]
Bifidobacteriaceae	0.624 ± 0.45 [a]	17.188 ± 12.735 [b]	7.894 ± 7.947 [ab]	13.577 ± 10.631 [b]
Campylobacteraceae	0.009 ± 0.024	0.093 ± 0.220	0.066 ± 0.151	0.294 ± 0.546
Clostridiaceae.1	0.273 ± 0.146	2.413 ± 3.231	5.509 ± 8.749	5.044 ± 4.581
Coriobacteriaceae	0.108 ± 0.039 [a]	0.039 ± 0.034 [b]	0.036 ± 0.041 [b]	0.023 ± 0.018 [b]
Corynebacteriaceae	0.032 ± 0.023	0.007 ± 0.011	0.020 ± 0.030	0.012 ± 0.023
Desulfovibrionaceae	0.098 ± 0.182	0.000 ± 0.000	0.003 ± 0.008	0.006 ± 0.014
Enterobacteriaceae	13.86 ± 5.97 [a]	23.48 ± 12.23 [ab]	19.51 ± 6.69 [b]	33.42 ± 11.99 [b]
Enterococcaceae	0.435 ± 0.707	0.145 ± 0.203	2.892 ± 6.293	0.542 ± 0.771
Erysipelotrichaceae	0.682 ± 0.387	4.080 ± 3.988	3.774 ± 4.950	2.766 ± 3.002
Family.XIII	0.062 ± 0.030 [a]	0.004 ± 0.008 [b]	0.000 ± 0.000 [b]	0.001 ± 0.003 [b]
Lachnospiraceae	6.327 ± 2.300 [a]	9.787 ± 6.180 [ab]	15.298 ± 9.544 [b]	4.962 ± 4.587 [b]
Lactobacillaceae	57.47 ± 8.72 [a]	28.74 ± 10.84 [b]	24.47 ± 5.71 [b]	31.13 ± 11.24 [b]
Micrococcaceae	0.140 ± 0.064	0.075 ± 0.074	0.071 ± 0.053	0.110 ± 0.105
Pasteurellaceae	0.582 ± 0.581	0.236 ± 0.235	0.456 ± 0.297	0.394 ± 0.446
Peptococcaceae	0.396 ± 0.182 [a]	0.006 ± 0.015 [b]	0.015 ± 0.019 [b]	0.007 ± 0.021 [b]
Peptostreptococcaceae	0.747 ± 0.485	0.471 ± 0.262	0.543 ± 0.108	0.640 ± 0.379
Porphyromonadaceae	1.242 ± 1.153	5.924 ± 9.747	9.826 ± 15.228	2.055 ± 5.475
Prevotellaceae	2.136 ± 1.540 [a]	0.014 ± 0.016 [b]	0.011 ± 0.018 [b]	0.028 ± 0.060 [b]
Rikenellaceae	0.034 ± 0.039 [a]	0.001 ± 0.004 [b]	0.000 ± 0.000 [b]	0.001 ± 0.003 [b]
Ruminococcaceae	3.242 ± 0.743 [a]	0.135 ± 0.147 [b]	1.610 ± 2.622 [b]	0.406 ± 0.665 [b]
Streptococcaceae	2.118 ± 0.620 [a]	0.510 ± 0.316 [b]	0.586 ± 0.156 [b]	0.643 ± 0.450 [b]

[1] Data are means ± SD (n = 6 to 8 per group). [2] Within a row, values followed by different letters (a,b,ab) differ significantly ($p < 0.05$).

In the cecum, the concentration of total end products increased in all OS groups compared to CTL (Figure 2). This was mainly due to an increase in SCFA concentration, which only reached statistical significance in the case of FOS and also an increase in lactate concentration in the case of αGOS.

Increases in total SCFA reflected acetate increases which were significant for both FOS and GOS/In groups, and paralleled significant decreases in pH values (Table 4). In addition, OS supplementation shifted microbiotal activity, as evidenced by significant changes in the relative proportions of acetate (93.8 ± 4.6, 93.1 ± 4.1, and 95.4 ± 2.9% for FOS, GOS/In and αGOS, respectively *versus* 86.3 ± 4.5% for CTL) and propionate (5.4 ± 4.6, 5.2 ± 3.4, and 3.6 ± 2.9% for FOS, GOS/In and αGOS, respectively versus 10.7 ± 3.0% for CTL). Concentration and relative proportions of butyrate—which is scarcely produced in the neonatal stage—were not affected significantly by supplementation.

Table 4. Concentration (mM) of major short chain fatty acids (SCFA) in cecocolonic contents at PND 14/15.

Treatment	Acetate	Propionate	Butyrate	pH
CTL	3.17 ± 1.05 [1,a,2]	0.39 ± 0.16	0.07 ± 0.04	6.9 ±0.3 [a]
GOS/In	5.82 ± 1.32 [b]	0.33 ± 0.21	0.10 ± 0.09	6.3 ±0.2 [b]
αGOS	5.69 ± 1.77 [ab]	0.28 ± 0.27	0.05 ± 0.00	6.1 ±0.2 [b]
FOS	8.00 ± 2.94 [b]	0.47 ± 0.37	0.06 ± 0.04	6.2 ±0.2 [b]

[1] Data are means ± SD (n = 7 to 8 per group). [2] Within columns, values followed by different letters (a,b,ab) differ significantly at $p < 0.05$.

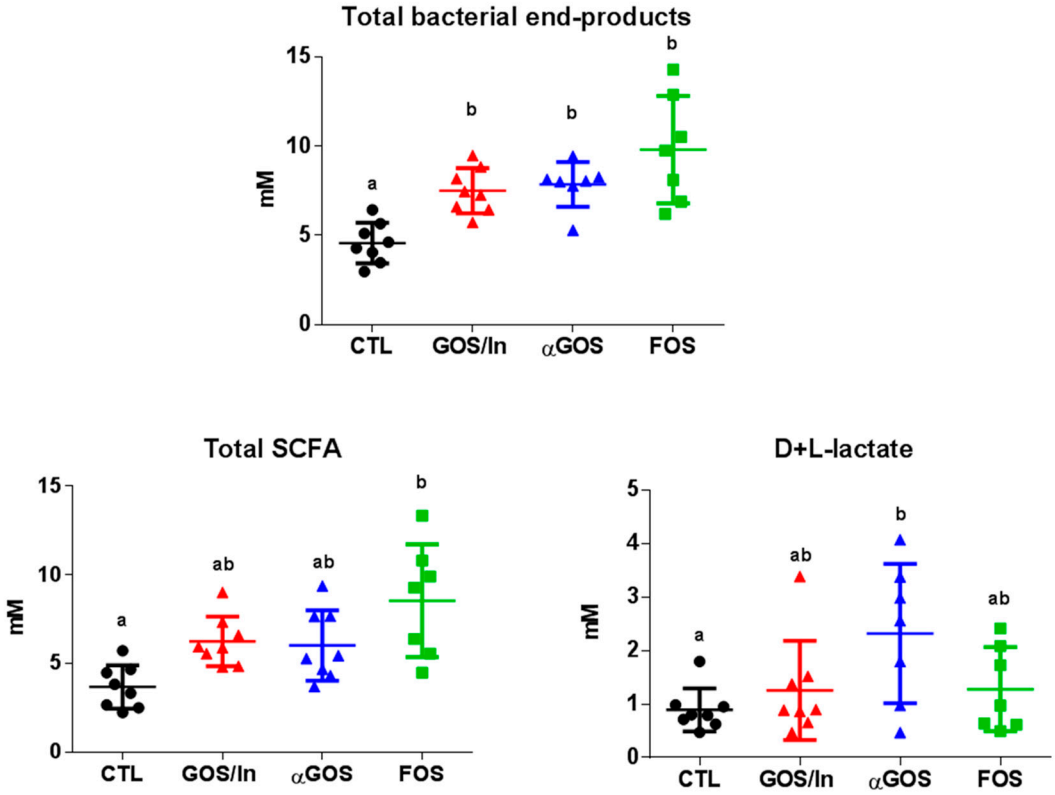

Figure 2. Cecocolonic concentrations of fermentation end-products. Individual, mean and SD values are plotted ($n = 7$ to 8 per group). Different letters indicated significant difference ($p < 0.05$) between groups.

3.2.2. OS Supplementation Modified both Differentiation and Activity of the Neonatal EEC

In the ileum, a profound effect on the enteroendocrine lineage was induced by neonatal OS supplementation, as revealed by a significant decrease in *Neurog3* expression in the OS groups compared to CTL, whereas, an early expressed marker in the commitment secretory lineage (*Atoh1*) was not affected significantly (Figure 3). The related expression of genes specifically implied in the differentiation of EECs (*Pax4* and *Pax6*) decreased significantly in OS supplemented groups compared to CTL, whereas expression of *Foxa1* did not vary between the groups. Similar to *Pax4* and *Pax6*, *Neurod1* expression decreased in OS groups compared with CTL, but this did not reach statistical significance for FOS. Regarding the expression of gene coding for peptides produced by mature L-cells, *Pyy* increased significantly in OS groups compared to CTL. At the same time, despite a 2-fold increase in *Gcg* expression in the OS groups compared to CTL, this effect was not statistically significant due to the widely varying expression between samples.

In the proximal colon, the impact of OS supplementation was much more moderate and their only significant effect was a decrease in the expression of *Pax4* (Figure S3).

Along with this profound remodeling in the expression of markers of L-cell differentiation, the number of GLP-1/ChgrA positive cells, i.e., mature EECs, was higher in the ileum of pups from OS groups compared to CTL but only reached statistical significance for villi (Figure 4A–C).

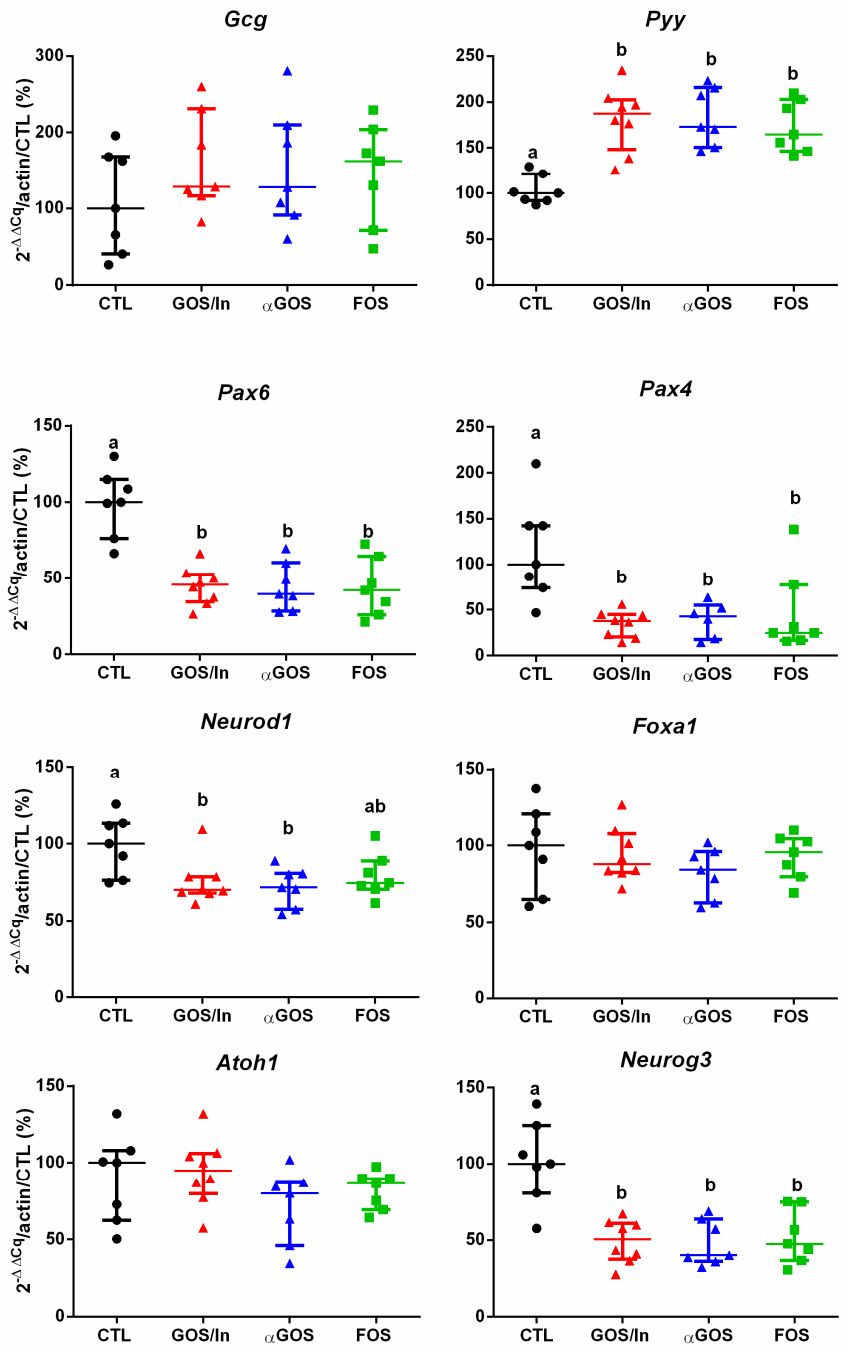

Figure 3. Relative expression of genes implied in the endocrine lineage and in L-cells differentiation in the ileum. Different letters indicate significant difference between groups ($p < 0.05$). Data are fold-change expressed in % of CTL group. Individual values, median with interquartile range are plotted ($n = 7$ to 8 per group).

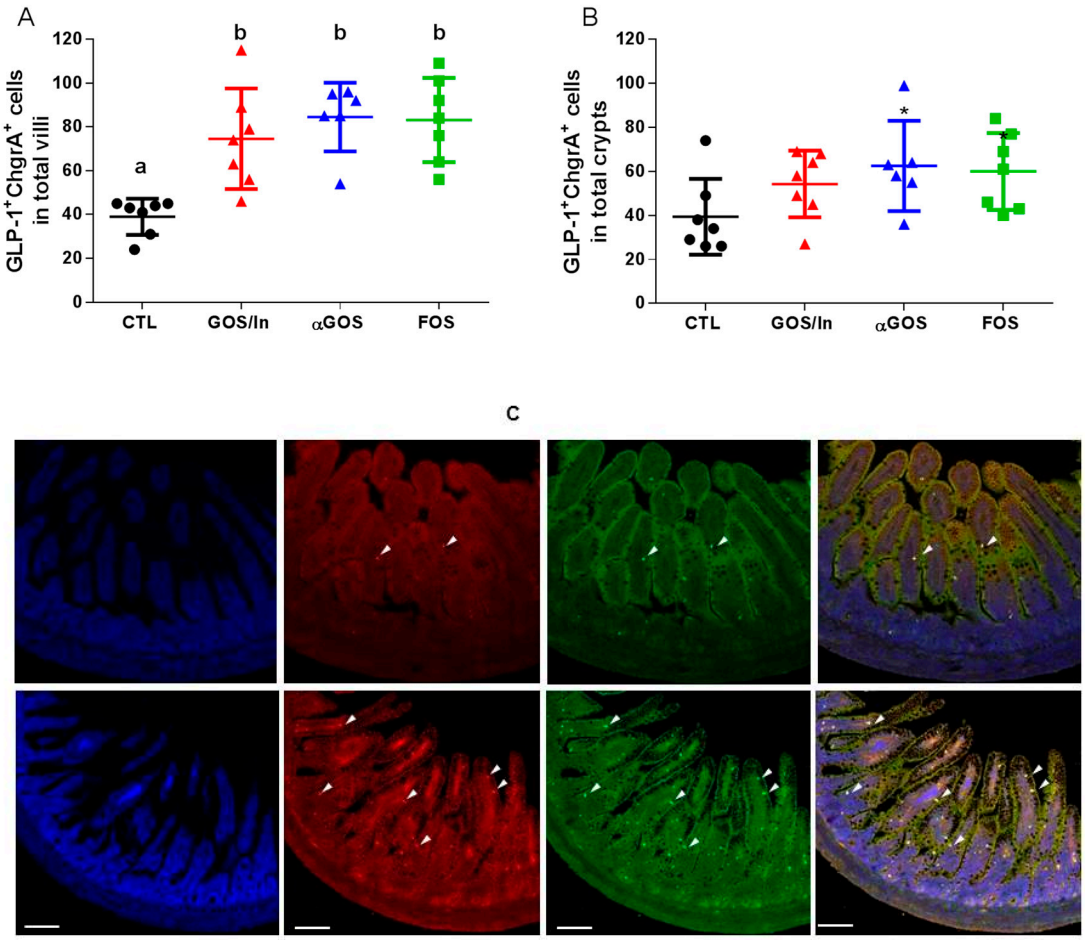

Figure 4. Effect of oligosaccharides (OS) supplementation on the density of GLP-1 cells in ileum: (**A**) in villi (**B**) in crypts. Different letters indicate significant differences among groups ($p < 0.05$); Individual, mean and SD values are plotted (n = 6 to 7 per group). (**C**) Representative images of immunofluorescence in ileal sections from a control solution (CTL) (top) and αGOS groups (down), arrows indicate positive fluorescence in cells: blue (DAPI, nuclei staining), red (GLP-1 cells), green (ChrgA cells) and merge (GLP-1/ChrgrA cells). Bars indicate 100 µm.

In agreement with this rise in the number of mature enteroendocrine cell (EEC), plasma concentrations of GLP-1 (Figure 5A) and PYY (Figure 5B) were significantly increased by all the neonatal OS supplementations, as compared with CTL.

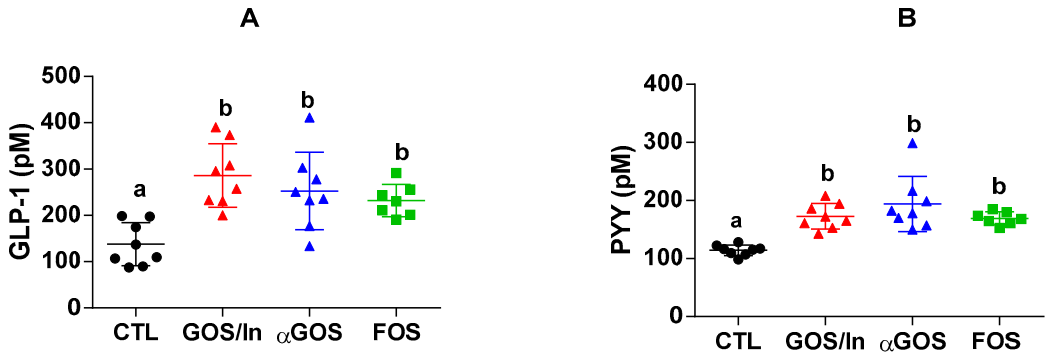

Figure 5. Plasma concentration of (**A**) Total GLP-1; (**B**) Total PYY at PND 14/15. Different letters indicate significant differences among groups ($p < 0.05$). Individual, mean and SD values are plotted (n = 7 to 8 per group).

Significant positive associations between plasma concentrations of GLP-1 and PYY and the ileal expression of their respective genes were evidenced (Figure 6A). Conversely, these plasma concentrations as well as the density of GLP-1 secreting cells, were inversely correlated with expressions of *Neurog3*, *Neurod1*, *Pax4*, and *Pax6*. With respect to associations between microbiota and EEC descriptors (Figure 6B), only some of the differentiating factors (*Pax4*, *Neurod1*, *Pax6* and *Neurog3*) exhibited significant positive correlations with the abundance of some bacterial families corresponding to those the abundance of which was significantly reduced by OS, except for Prevotellaceae. For these factors, the sole negative correlation was that between *Neurod1* and abundance of Clostridiaceae.1. Conversely, the PYY and GLP-1 plasmatic concentrations, EEC densities and *Pyy* expression, but not *Gcg* expression, were negatively correlated with the same families including Prevotellaceae.

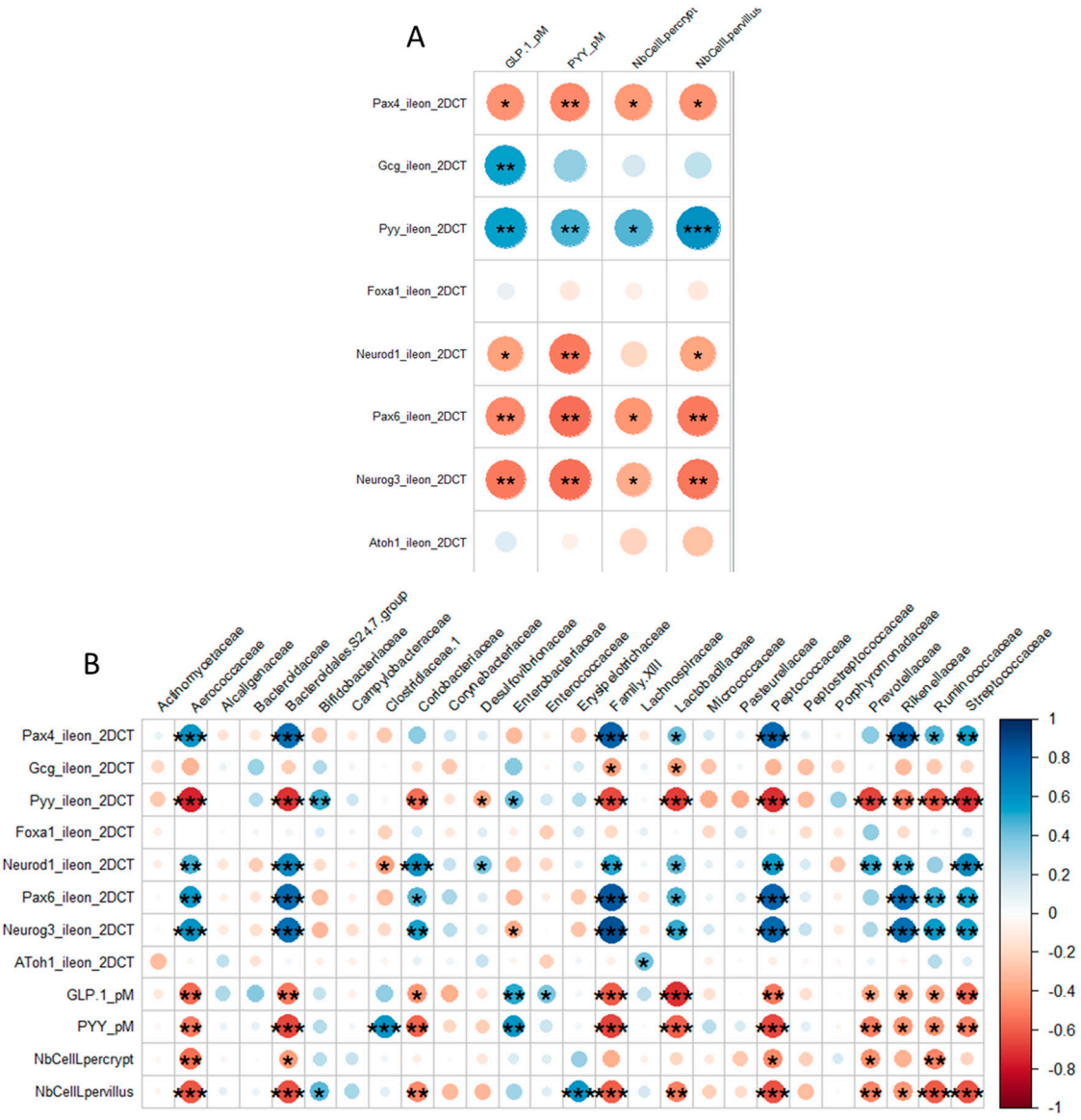

Figure 6. Correlograms within EEC descriptors (**A**) or between these descriptors and the relative abundances of main bacterial families (**B**). Positive correlations are displayed in blue and negative correlations in red. The intensity of the color and the size of the circles are proportional to the correlation coefficients. Asterisks indicate the level of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). On the right of the correlogram, the color legend shows the correspondence between correlation coefficients and colors.

Overall, these results indicate that OS supplementation profoundly modulates neonatal microbiota in terms of both its composition and its fermentative activity, with repercussions not only in the cecocolon but also, as exemplified with FOS, in the ileum. An increased density of ileal L-EECs and their secreted anorectic hormones, GLP-1 and PYY, were observed and unexpectedly the expression of transcription factors beyond the stage of secretory cell engagement (Atoh1) was inhibited at the same time. Whether this strong impact of early OS supplementation on satiety peptide-related EECs could last into later life and affect eating behavior was investigated further.

3.3. Neonatal OS Supplementation Had No Significant Long-Term Consequences

3.3.1. Neonatal OS Supplementation Did Not Significantly Program Enteropeptide Production or Eating Behavior in Adulthood

To investigate the long-term effect of neonatal supplementation of OS on nutrient sensing in EECs, once pups reached adulthood, we studied the release of GLP-1 and PYY in response to both a 20-min test meal (PND 74/76) and an oral bolus of glucose (PND 124/126) after 16 h of fasting.

No significant differences were observed between groups in the amount of food consumed during the 20-min test meal (Figure 7A). In response to this meal, the plasma concentration of GLP-1 increased immediately after refeeding and returned to pre-prandial level 120 and 180 min later (Figure 7B). The total amount of GLP-1 secreted during this period, quantified by AUC, did not differ significantly between the groups (Figure 7C). PYY secretion did not show any postprandial peak or significant differences between the groups (data not shown).

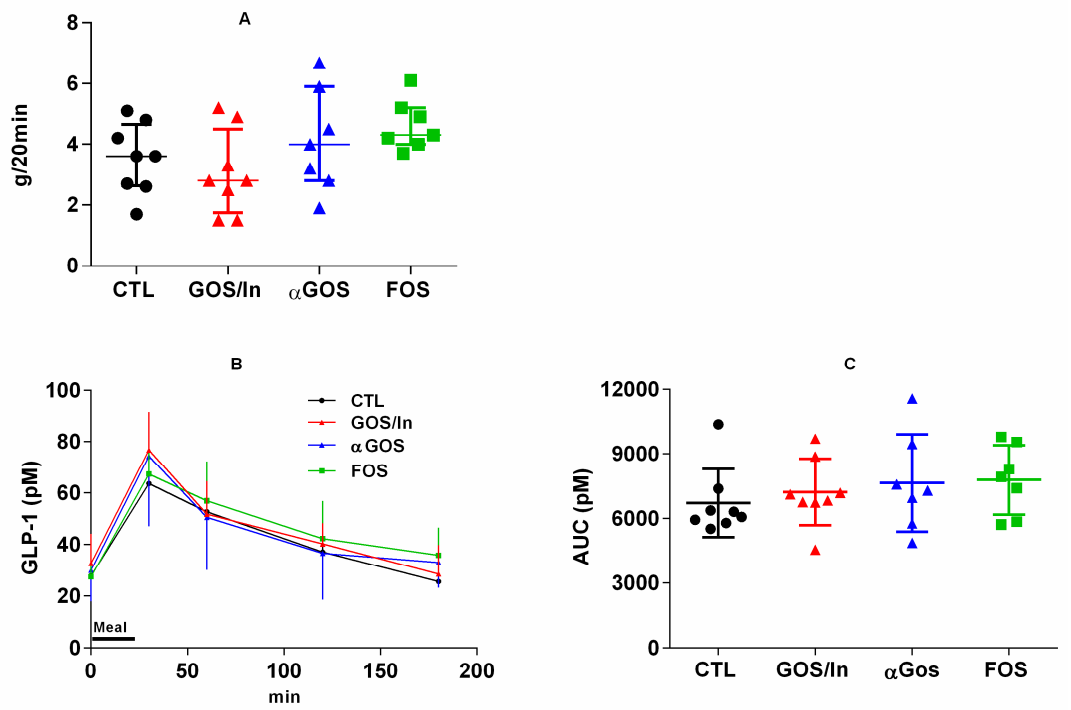

Figure 7. Fasting-refeeding test. (**A**) Food intake measured during refeeding (20 min-meal); (**B**) Plasma concentration of total GLP-1 measured during the 3h-kinetic follow-up (means ± SD); (**C**) Total amount of GLP-1 secreted during the 0-180min period expressed as AUC. Individuals, means and SD are plotted. (n = 7 to 8 per groups).

Similarly, at PND 124/126, plasma concentrations of GLP-1 (CTL: 34.4 ± 13.5; GOS/In: 38.6 ± 28.6; αGOS: 28.9 ± 10.0 and FOS: 37.9 ± 20.6 pM) and PYY (CTL: 84.7 ± 4.0; GOS/In: 88.7 ± 7.8; αGOS: 91.4 ± 7.3 and FOS: 91.5 ± 5.8 pM) measured 2h after an oral bolus of glucose did not show any significant difference between groups.

To investigate the long-term effect of a neonatal supplementation of OS on subsequent eating behavior, we followed up the food consumption from weaning to adulthood, performed a refined analysis of feeding pattern using physiological cages from PND75 to PND100 and assessed the preference for sugar taste between PND109 and PND111.

The analysis of food consumption during development, expressed per Kg of body weight to allow for strict comparison, only revealed a single significant difference which occurred at PND32 between animals from the FOS and CTL groups (Figure 8), an observation which indicates that neonatal supplementation with OS did not greatly influence the subsequent food intake in our experimental conditions.

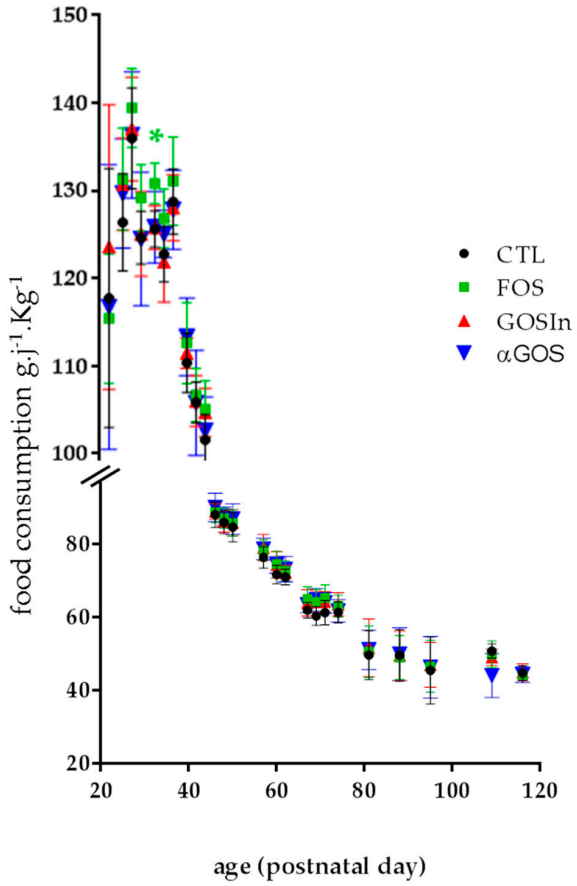

Figure 8. Daily consumption of food in the post-weaning stage, expressed as kilograms of bodyweight. The asterisk indicates a significant difference between FOS and CTL groups ($p < 0.05$). Data are means ± SD ($n = 7$ to 8 by group and day).

This absence of effect on daily food consumption was confirmed by a detailed analysis of food consumption: we observed no significant difference in meal patterns among the groups (food intake, food intake per meal, number and duration of meals, latency to eat the first nocturnal meal, satiety ratio and ingestion rate), whatever the period of measurement (total 20 h period of measurement, diurnal period (8 h) or nocturnal period (12 h) (Figure 9 and Figure S4).

In the sweet taste preference test, there was no significant difference between groups in terms of the consumption of saccharin solution expressed as a percentage of daily beverage intake, regardless of the day of testing (Figure 10). Strikingly, the preference for sweet taste for the GOS/In group did not reach statistical significance on the first day of the test, in contrast to the FOS and αGOS groups. However, this preference did not persist on day 2, contrary to what was observed for the CTL group. This suggests that neonatal supplementation with OS slightly reduced the persistence of sweet preference in adulthood.

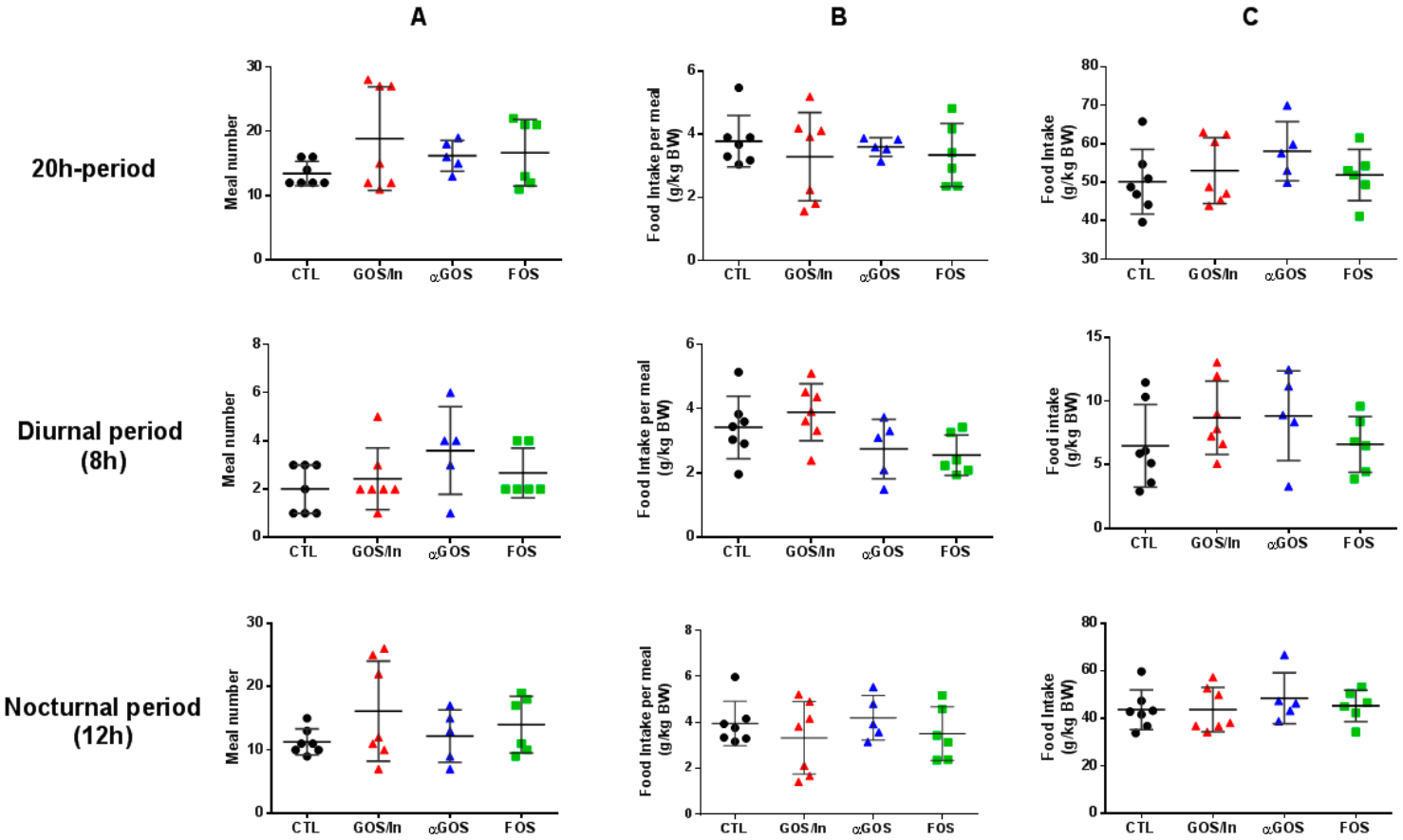

Figure 9. Feeding patterns illustrated by (**A**) Meal number; (**B**) Food intake per meal; (**C**) Food intake during the considered period analyzed in physiological cages at PND 75–100 (n = 5–7 per group). BW, bodyweight. Individuals, means and SD are plotted (n = 5 to 7 per groups).

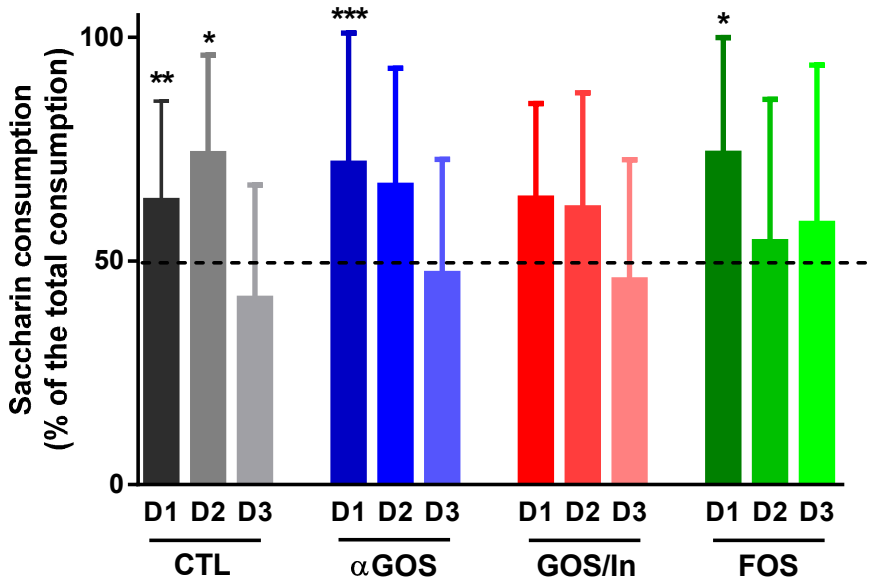

Figure 10. Preference for sweet taste. Data are means ± SD (n = 7 to 8). Asterisks represent significant preference as compared with no preference (i.e., 50%, dotted line): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.3.2. Neonatal OS Supplementation Did Not Significantly Program Adult Intestinal Microbiota

At adult age (PND 124/126), no significant differences were observed between treatments with respect to the raw number of sequences obtained, percentages of sequences kept after quality filtering, or alpha-diversity indexes (data not shown). Similarly, β-diversity analysis (Figure S5), principal component analysis on OTU abundances (Data not shown) and comparisons of the cumulated relative abundances at family level (Figure 11) failed to show any significant difference between cecal samples with respect to neonatal supplementation. Finally, neither ileal nor cecal concentrations of SCFA showed significant differences between the groups (Table S2).

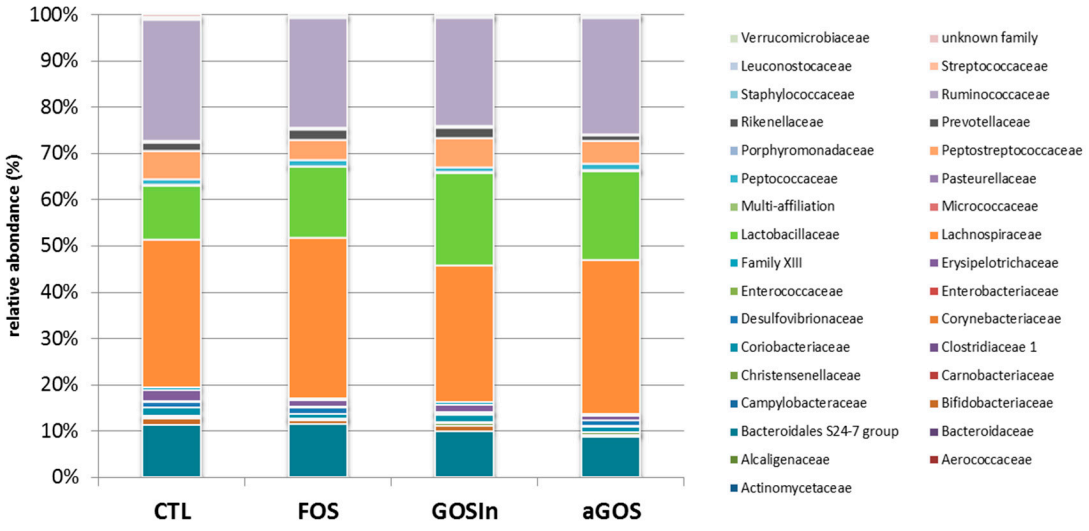

Figure 11. Impact of postnatal OS-supplementation on cecal microbiota composition at PND124/126: families distribution expressed as the average of cumulated relative abundances (n = 7 to 8 per group).

Overall, these data did not reveal that neonatal OS supplementation had any programming effect on adult microbiota.

4. Discussion

Considering that the regulation of feeding behavior could be programmed from the beginning of life and controlled by intestinal microbiota, we hypothesized that modifications to the neonatal microbiota could program adult feeding behavior. We therefore checked the ability of prebiotic-induced intestinal microbiota modulations to affect the maturation and functioning of L-EECs in suckled male rats, then assessed whether this resulted in delayed alterations in eating behavior and the secretion of GI peptides in adulthood. The observed effects are specifically attributable to OS since we adjusted the compositions of the administered solutions by taking into account the digestible sugar contents of commercial OS sources. In this study, we show that neonatal supplementation with 3 different OS strongly impacts cecocolonic microbiota, GLP-1 cell density in the ileum, and the production of satiety-related peptides during the neonatal period, but does not induce any significant enduring effect in adulthood on either eating behavior or gut peptide secretion.

The validity of this statement is obviously limited to our operating conditions which represent both strengths and limitations for our study.

Limitations include the fact that we only studied males in order to avoid the already described fluctuations in food intake throughout the estrous cycles [41], and did not characterize every components of eating behaviour such as motivation. However, we believe that the numerous components investigated allow for consideration of both its homeostatic and hedonic elements. We did not investigate immediate impact of OS supplementations on feeding behavior to avoid the recognized stress induced in pups by separation from the mother which would have been required to quantify milk intake either by gravimetric [42] or deuterated water turnover methods [43]. We did consider moreover whether neonatal prebiotic supplementation having an impact on the pups' eating behavior was beyond the scope of the programming of adult eating behavior. Nevertheless, we reported a transitory reduction in BW gain in FOS and GOS/In groups between PND6 and PND10 which suggests that the reducing impact of OS on food intake may also operate in the neonatal period.

Inversely, our study has three major advantages: the combination of hormonal, behavioral and microbiological analyzes; the minimizing of the influence of lactating mother influence by supplementing pups from the same litters with the different OS, and finally the use of OS doses comparable to those actually consumed by toddlers.

4.1. Neonatal OS Supplementation Affected Intestinal Microbiota Despite Its Immaturity

Corroborating our previous findings based on a non-exhaustive analysis of the microbiota [24], and in concurrence with several in vivo and in vitro studies investigating the impact in adulthood of OS (including those of the αGOS [44]) on intestinal microbiota, in humans and animals (e.g., [45,46]) and in human infants (see [32] for review), all the oligosaccharides used here dramatically affected neonatal microbiota in rat pups. This confirms that the prebiotic properties previously demonstrated in adult rodents (e.g., [30,45,46]), also operate in neonatal pups despite the immaturity of the microbiota at this stage of development [37].

In addition to these changes in composition and the reduction in microbiota richness, our neonatal OS supplementations also modified the activity of the microbiota by stimulating the production of acetate and lactate at the expense of that of propionate. This decrease in propionate concentration stands out from what is observed in adult rats, for which GOS and FOS are frequently reported as being particularly stimulating for propionate and/or butyrate production (e.g., [34,47]) and could be related to the known progressive maturation of the microbiotal capacity to synthesis the different SCFAs in neonates [37,48]. The production of butyrate is therefore barely detectable before the day 16 of life in rats [37]. In any case, the neonatal OS supplementation we performed resulted in microbiota that differed greatly from that of unsupplemented animals, an observation which was a prerequisite for investigating the ability of neonatal microbiota modulation to program adult eating behavior or gut peptide response.

4.2. OS Supplementation May Stimulate Ileal EECs to Produce GLP-1/PYY While Acting in Feedback on Endocrine Precursors

Our results showed that neonatal OS supplementation had immediate effects on mature ileal GLP-1-cells by increasing the density in villi and the mRNA expression of *Gcg* and *PYY* leading to enhanced plasma concentrations in these two anorectic peptides. These new observations in neonatal rats are consistent with those reported in adult rats for FOS and GOS/In [33,34,49–51] and are, to our knowledge, reported here for the first time for αGOS. In one of these previous studies, this increased production of GLP-1 was related to a higher differentiation of *Neurog3*-expressing EEC progenitors into L-cells in the colon [50]. Here, we demonstrate a drastic down-regulation of endocrine lineage-devoted genes during OS supplementation, mainly in the ileum. This unexpected result is difficult to reconcile as an effect of OS on early endocrine precursors leading to the production of more L-cell subtypes.

Neurog3 marks the endocrine progenitors and is essential for generating new EECs [52]. Post-neurog3 differentiation and maturation of EECs is controlled by dynamics in transcriptional factors such *Neurod1*, *Pax4* and *Pax6* and many others (*Arx*, *Pdx1*, *Foxa1* and *Foxa2*). The hierarchy of these events is still poorly understood [53] and the extrinsic factors that may interplay remain largely unknown. For this study, the well-known effect of OS prebiotics in stimulating L-cells cannot simply be explained by the impact on endocrine precursors, as suggested in the above-mentioned study [50]. Since we know that *Neurog3* expression is restricted to immature proliferative cells, the decreased Neurog3 expression we observed in the ileum may instead reflect a feedback regulation to limit new EEC generation in response to OS supplementation. A similar observation (decreased duodenal Neurog3 and increased EEC density) was reported in a model of maternal deprivation [26]. These data and our own suggest that the postnatal environment affects the differentiation of EEC precursors but not the proliferation of progenitors, leading to increased EEC density. High levels of circulating GLP-1 have been previously attributed to the increased number of ileal L-cells in *Gcgr*-deleted mice, and this effect involved up-regulation of post-*neurog3* transcription factors, affecting the proliferation of L-cells precursors [54]. Here, the expression of these factors, i.e., *Neurod1*, *Pax4* and *Pax6*, was reduced in OS-supplemented groups with high circulating levels of GLP-1, suggesting a different mechanism in the increased density of L-cells. In this respect, it should be noted that although EECs are still classified according to their major/unique hormone product (as for example GLP-1 for L-cells), it is now acknowledged that EECs are multihormonal [53,55]. In particular, more recent data has demonstrated that mature differentiated EECs display hormonal plasticity, allowing them to change their hormonal products in response to extrinsic factors, such as bone morphogenic proteins (BMP) during their migration along the crypt-villus axis [56,57]. Thus, the increased L-cell density observed here may be the result of the direct effect of OS on this plasticity to produce more GLP-1, independently of early markers of EEC proliferation and differentiation. Interestingly, in this study, the production of CCK—a key early-satiety peptide—was not affected by the OS supplementation at PND14/15 (data not shown) reinforcing the specificity of the effect of OS on EECs in producing GLP-1 and PYY in a segment of gut where CCK is not predominantly produced. How OS can modulate both the identity of EEC subtypes and/or the expression of GI peptides by acting on extrinsic factors (such as villus-produced BMP) needs further investigation.

4.3. What are the Putative Mediators of the Massive Effect of Neonatal OS Supplementation on Ileal L-Cells?

Identification of the small intestine rather than the colon as a privileged site for the action of OS on transcriptional activity has been previously reported in studies involving adult animals [58,59]. Conventionalization of germ-free mice led to similar observations (e.g., [60]). However, a nutritional modulation by OS supplementation may have a different impact on ileal epithelium compared to the absence or presence of microbiota. For example, in the Arora's study [60], conventionalization of germ-free mice led to the down-regulation of GLP-1 secreting vesicle process in L-cells, whereas we observed an increase in GLP-1 and PYY production. These contrasting results may stem from either inter-individual variability, or more likely the great differences in age between the animals studied.

Nevertheless, our data raise the question of how OS modulation of microbiota could act on ileal L-cells. The well-known capacity of SCFA (mainly butyrate but also propionate or even, non-consensually, acetate) to stimulate PYY and/or GLP-1 production [61–63] seems inconsistent with our observation of an OS-impact mainly localized in the ileum, within the context of no propionate/butyrate synthesis.

Others potential mechanisms include acidification of the luminal milieu or changes in the pathogen-associated molecular patterns (PAMPs). Zhou et al. [61] showed that changes in pH from 7.5 to 6.5 induce *per se* an increase in *Gcg* expression by STC-1 cells in vitro. Apart from this, it is known that EECs have receptors for PAMPs (i.e., Toll-like receptors) (see [18] for review). This is of particular interest since it has been demonstrated that some bacterial strains elicit GLP-1 secretion through signaling agents of the Toll-like receptor system, as illustrated by the fact that a MyD88 blockade triggers GLP-1 secretion induced by bacteria [64].

4.4. OS impact on Eating Behavior, Usually Observed Simultaneously with Their Consumption, Does Not Seem to Be Programmable

Despite a certain disparity in the literature, possibly related to the heterogeneity in dosage or methodology, several studies have reported the beneficial effect of OS prebiotics—mainly fructans but also αGOS, on the eating habits of healthy adults [65,66] or overweight adults [67,68], such as feelings of reduced hunger, increased satiety or reduced energy consumption. Note that the existing literature does not establish whether this is also true in infants, who are frequently given prebiotic supplements. In concurrence with human data, decreased food/energy intake has been evidenced in adult rodents supplemented with fructans [49,51] or βGOS [34]. In both models, these effects have been related to SCFA production by colonic bacteria during OS supplementation. For each of the 3 main SCFAs, i.e., acetate, propionate and butyrate, it has been demonstrated that they reduce energy intake, particularly in rodent models of diet-induced obesity [69–71], although conflicting results are reported [72], probably dependent on the mode (orogastric [71,72], intraperitoneal [70], intracerebroventricular [70], colonic delivery via fermentable fibers [69,71], etc.) and duration (acute [69,70] vs. chronic [69,72]) of SCFA or SCFA precursors administration. In humans, this hypothesis has been substantiated for both acetate and propionate by numerous studies focusing on appetite-related parameters (see [73] for review) as well as observations of reduced hedonic response to high-energy foods regulated in striatum [74] or reduced energy intake following the administration of propionate precursors in overweight adults [75]. How these SCFA regulate appetite directly at hypothalamic level [70] or via a vagal-dependent mechanism [71,72], whether or not implicating an enhanced intestinal satiety peptide (GLP-1 and PYY) secretion following SCFA interaction with FFAR receptors on L-cells is still a matter of experimental research in animal models and clinical trials in humans [18].

Since the perinatal environment [6,9,24,26] appears to have a long-lasting impact on each of the microbiota-EEC-brain axis actors, we had assumed that early modulation of the microbiota associated with changes in EECs could program eating behavior, a hypothesis which has remained unexplored until now. However, this hypothesis could not be corroborated in this study as adult feeding behavior did not seem to be significantly affected by early supplementation with OS, which nonetheless increased total SCFA, along with increased release of GLP-1 and PYY and L-cell density at the end of supplementation. This lack of eating behavior programming indicates that none of the presupposed events (i.e., programming of EEC or vagal sensitivity and/or microbiota programming) occurred under the test conditions. In fact, no difference in the expression of *c-Fos* was observed in the nucleus of the solitary tract in the rat's brainstem 2 h after administering a bolus of glucose in adult rats (data not shown). It therefore seems that depending on the nature and intensity of the perinatal stressor (maternal protein restriction [9], maternal deprivation [26] or postnatal modulation of microbiota by OS) the long-lasting impact is not systematic. For the microbiota, the lack of programming could be related to an inadequacy in the timing for applying the modulation, as discussed below.

4.5. Is Programming of the Microbiota Subject to Particular Timing?

In this study, we did not observe any programming effect of neonatal OS supplementation on adult microbiota. This result is in line with what we had previously observed for FOS [24] but contradicts the small-scale programming found after neonatal supplementation with GOS/In in this same study. This discrepancy may result from the difference in methods used to analyze the composition of the microbiota, even if it is counterintuitive, since the 16S rDNA sequencing used here is more exhaustive than the qPCR used previously. As this impact was minor, it may also not have been possible to reproduce under our new experimental conditions, i.e., a new batch of animals, a different room at our animal facility, or even a slight difference in the composition of the semi-purified diets we used, since all these parameters are known to affect the microbiota of laboratory animals (see [76] for review).

The disappearance of this nonetheless drastic effect in our animals at the end of supplementation raises the question of what is the most favorable period for sustainable modulation of the composition of the microbiota. In our experimental protocol, prebiotic supplementation was applied for a short postnatal period and ended before the onset of solid food consumption, whereas studies reporting programming effects for early supplementation with OS on the subsequent composition of the microbiota were based on longer-term supplementation, ranging from the prenatal period (i.e., supplementation of gestating mothers) to complete weaning and even beyond [77,78]. Whether the supplementation we applied was either not early enough, not late enough or not for a long enough time is difficult to establish on the sole basis of this comparison. However, in a study by Fugiwara et al. [77], a difference in adult microbiota composition was observed only in mice offspring that were supplemented with FOS beyond weaning. Whether this was also true in the Le Bourgot et al. study [78] cannot be evaluated since all piglets were supplemented with FOS for a few weeks after weaning. From this, it can be assumed that to be lastingly effective, prebiotics must be able to exert their microbiotal effect after full weaning, thereby controlling the impact of new bacterial sources and changes in dynamics of bacterial populations that result from the switch from maternal milk to solid food. Such a switch has been associated with dramatic changes in microbiota composition and activity both in humans [79] and rats [37]. This hypothesis would explain why the early-life events that are known to affect neonatal microbiota composition (i.e., birth mode, infant feeding etc.) are not associated with significant variations in adult microbiota composition [80], but strict comparisons between the time windows for supplementation are required for this to be validated.

4.6. All OS Studied Performed Similarly Despite Differences in Their Chemical Characteristics

In our study, the 3 OS studied led to comparable results in terms of both microbiotal impact and physiological repercussions. With regard to microbiotal changes, the observed modifications, in particular the acidification of the contents, the less diversified production of SCFA and the reduced richness of microbiota suggest that OS delays bacterial diversification. This is similar to what is supposed to happen in breast-fed babies compared with babies fed with unsupplemented formula [81]. The similarity is quite surprising in that the chemical nature of the constituent monomers and the pattern of glycoside linkages in different OS products are expected to influence the ability of individual bacteria to grow on them (see [31,82] for reviews). However, our results are consistent with Harris et al.'s findings [83] that the orientation of glycoside linkage is not a main driver of the SCFA production profile. When this chemical difference could act, it would primarily modulate the proportion of butyrate, an SCFA weakly produced in our immature animals. In addition, they also agree with the similarities of microbiotal impacts reported between βGOS and FOS on the one hand [30], and between αGOS and βGOS on the other [44].

Thus, our study confirms the prebiotic character of αGOS and, in addition, extends the well-known activity of FOS and GOS/In as secretagogues of satiety enteropeptides to this new prebiotic, a finding which is in accordance with the satietogenic effect described in humans [67].

5. Conclusions

In conclusion, our study depicts that the ability of the OS to modulate EECs as previously described in adults also operates in the neonatal period, despite the immaturity of the microbiota at this time. This observation therefore calls into question the nature of the mediators actually involved, as supposed so far. In addition, our in-depth study of the impacts of the OS on the genes regulating the differentiation of EEC precursors queries the current understanding of the ontogenesis of these cells.

Finally, our results do not demonstrate any programming impact of OS either on EECs and food consumption or on the constitution of the adult microbiota. If this holds true for humans, it is reassuring since this study concerns types and dosages of OS mimicking some of those commonly prescribed in formula for toddlers.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/9/1967/s1, Figure S1: Postnatal growth of suckling rats in the different groups of OS supplementation, Table S1: Primer sequences, Figure S2: Growth of weaned rats until adulthood, Figure S3: Relative expression of gene implied in the endocrine lineage and in L-cells differentiation in the colon. Figure S4: Supplemental parameters of meal pattern. Figure S5: Hierarchical clustering of phylogenetically informed distance matrix computed using the unweighted UniFrac metric for cecal contents collected at PND 124/126. Table S2: Concentration of SCFA in ileal and cecal contents at PND 124/126.

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Annexe 4. Publication #3.

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ORIGINAL RESEARCH
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Maternal Microbiota Transfer Programs Offspring Eating Behavior

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Understanding the link between mother's obesity and regulation of the child's appetite is a prerequisite for the design of successful preventive strategies. Beyond the possible contributions of genetic heritage, family culture, and hormonal and metabolic environment during pregnancy, we investigate in the present paper the causal role of the transmission of the maternal microbiotas in obesity as microbiotas differ between lean and obese mothers, maternal microbiotas are the main determinants of a baby's gut colonization, and the intestinal microbiota resulting from the early colonization could impact the feeding behavior of the offspring with short- and long-term consequences on body weight. We thus investigated the potential role of vertical transfers of maternal microbiotas in programming the eating behavior of the offspring. Selectively bred obese-prone (OP)/obese-resistant (OR) Sprague-Dawley dams were used since differences in the cecal microbiota have been evidenced from males of that strain. Microbiota collected from vagina (at the end of gestation), feces, and milk (at postnatal days 1, 5, 10, and 15) of OP/OR dams were orally inoculated to conventional Fischer F344 recipient pups from birth to 15 days of age to create three groups of pups: F-OP, F-OR, and F-Sham group (that received the vehicle). We first checked microbiotal differences between inoculas. We then assessed the impact of transfer (from birth to adulthood) onto the intestinal microbiota of recipients rats, their growth, and their eating behavior by measuring their caloric intake, their anticipatory food reward responses, their preference for sweet and fat tastes in solutions, and the sensations that extend after food ingestion. Finally, we searched for correlation between microbiota composition and food intake parameters. We found that maternal transfer of microbiota differing in composition led to alterations in pups' gut microbiota composition that did not last until adulthood but were associated with specific eating behavior characteristics that were predisposing F-OP rats to higher risk of over consuming at subsequent periods of their life. These findings support the view that neonatal gut microbiotal transfer can program eating behavior, even without a significant long-lasting impact on adulthood microbiota composition.

Keywords: 16S rDNA sequencing, food preference, motivation, obesity, DOHaD (development origins of health and disease), OP rat

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INTRODUCTION

Obesity remains a major public health concern since its prevalence is still on the rise in specific age groups, particularly in the 5–19-year-old group (Who, 2020), and a third of French women of childbearing age are either overweight or obese (Matta et al., 2016). Obesity in this age range is of particular concern since there is overwhelming evidence that being born to an obese mother increases the risk for the child to develop excess adiposity (Weng et al., 2013; Juonala et al., 2020). Birth weight and weight gain during infancy are important factors in the subsequent development of obesity and chronic diseases associated with excess of adiposity, such as type 2 diabetes or cardiovascular disease (The GBD 2015 Obesity Collaborators, 2017), which expose the future adult to a higher risk of death (Abdelaal et al., 2017). Excess intake of calories and alteration of the reward system regulation, associated with low physical activity and consumption of food of low quality, are among the main determinants of the vast majority of cases of obesity not associated to genetic polymorphism. Understanding the link between regulation of the child's appetite and his/her mother's obesity is a prerequisite for the design of successful preventive strategies. We propose in the present paper to investigate the causal role of transmission of maternal microbiotas, in obesity, as microbiotas differ between lean and obese mothers, are the main determinants of offspring gut colonization, and the resulting intestinal microbiota could impact feeding behavior of the offspring.

Most studies comparing the fecal microbiotas of obese or overweight subjects with lean ones concluded that there were differences in composition (for review, see Gerard, 2016). Such differences were reported in pregnant women (Collado et al., 2008; Dreisbach et al., 2020) regarding not only intestinal microbiota but also the microbiota associated with breast milk (Cabrera-Rubio et al., 2012; Lundgren et al., 2019), and vaginal mucosa (Si et al., 2017). Despite some discrepancies (e.g., Stanislawski et al., 2017), differences were also found among descendants depending on whether they were born to obese or lean mothers (for review, see Dreisbach et al., 2020). Such a finding is consistent with the major contribution of maternal microbiotas to the initial colonization of the infant's gastrointestinal tract (Ferretti et al., 2018). Bacterial colonization of the human sterile gut begins during passage though the birth canal and undergoes distinct phases of progression during the first months of life to reach a richness and diversity almost similar to that of adults by 3 years of age, even though complete maturation of the gut microbiota might take longer (Stewart et al., 2018; Derrien et al., 2019). Bacterial strains tracking between maternal sources and infant gut microbiotas has evidenced that all maternal microbiotas provide inocula for this colonization process with vertical transfers demonstrated for vaginal bacteria (Matsumiya et al., 2002), fecal bacteria (Backhed et al., 2015; Asnicar et al., 2017; Ferretti et al., 2018), as well as for bacteria associated to breast milk (Martin et al., 2012; Jost et al., 2014; Pannaraj et al., 2017). Among these strains, some were only transiently detected, particularly for species typical of the vaginal microbiota which become undetectable by 1 week of age (Ferretti et al., 2018).

Because of its vast biochemical potential superimposed on that of the host, the gut microbiota can contribute to the host's phenotype, both by interacting with its organ functions in adulthood or by programming organ development in the infant. Previously, the gut microbiota has been shown to be involved in improving the energy yields from food, or modulating energy balance (for review, see Heiss and Olofsson, 2018), or in the accretion of the adipose tissue in animal models (Backhed et al., 2004) and humans (Le Roy et al., 2019). However, microbial communities are also increasingly recognized as modulators of complex animal behaviors such as social interactions, stress-induced behavior (anxiety, depression), as well as cognitive behavior (learning and memory tasks) (for review, see Vuong et al., 2017). The contribution of gut microbiota in feeding behavior (appetite or eating regulation) has been first evidenced by demonstrating differences in food intake between germ-free and conventional animals (Backhed et al., 2004; Rabot et al., 2010) and a decrease in food intake following the intraperitoneal injections of intestinal *Escherichia coli* ClpB protein in mice (Breton et al., 2016). Such contribution is supported by the fact that germ-free status is associated with an altered expression of several genes involved in food intake regulation, such as *bdnf* or *gcg* in brain (Diaz Heijtz et al., 2011; Schele et al., 2013)or the hypothalamic inhibitory factor receptor GABA-R, whose expression is restored by supplementation of a particular bacterial strain (*Lactobacillus rhamnosus*, strain JB-1) (Bravo et al., 2011). Lastly, prebiotic supplementation has been shown to modify the balance between limbic and hypothalamic gene expression involved in food reward and food intake (Delbes et al., 2018). However, the ability of early gut microbiota to program the eating behavior of adults is poorly studied (van de Wouw et al., 2017). This issue clearly deserves further study given the large body of evidence showing that eating behavior and the neural circuits involved in its control, i.e., the vagus nerve, hypothalamus, and reward circuits, can be programmed early in life through nutrition and the environment factors (Coupe et al., 2010; Ross and Desai, 2014; Ndjim et al., 2017; Paradis et al., 2017).

We therefore investigated the potential role of microbiota transfer from mother to child as a route through which the eating behavior of the offspring can be programmed.

To test such hypothesis, we used selectively bred obese-prone (OP)/obese-resistant (OR) Sprague-Dawley rats. Of particular relevance to our hypothesis, males from these strains exhibit differences in their cecal microbiota (Obanda et al., 2018). We collected feces-, vagina-, and milk-associated microbiota from OP/OR dams before transferring them to conventional Fischer F344 recipient pups between birth to 15 days of age, so as to form three groups of pups: F-OP, F-OR, and F-Sham groups (that received the vehicle). As a prerequisite, we checked that inoculas differed in their microbiotal composition according to the genetic background of the donors and assessed the impact of transfer onto the gut microbiota of recipient rats at PND21, PND60, PND130, and PND200. We then characterized the growth and eating behavior of the three groups, from birth to adulthood, by measuring their calorie intake, their anticipatory food reward

responses, the taste perception or gustation during ingestion of sweet and fat solution, and the sensations that extend after food ingestion. We finally searched for correlations between microbiota composition and food intake parameters.

MATERIALS AND METHODS

Ethics Statement

All experiments were conducted in accordance with the European Union regulations for the care and use of animals for experimental procedures (2010/63/EU). Protocols were approved by the local Committee on the Ethics in Animal Experiments of Pays de la Loire (France) and the French Ministry of Research (APAFIS#6617-2016072916395797-v6). Animal facility is registered by the French Veterinary Department as A44276.

Inocula

Donor Mothers

Obese-prone [Crl:OP(CD), OP] and obese-resistant [Crl:OR(CD), OR] 7-week-old female rats derived from those initially selected by Levin (Levin et al., 1997) were obtained from Charles River Labs (Kingston, NY, United States). Following 2 weeks adaptation to a standard diet (A03, Safe Diet, Augy, France), females were fed a high energetic diet (HED) (58V8, TestDiet, Richmond, United States) (for more details, see **Supplementary Table 2**) for 4 weeks then mated with Crl:OR(CD) or standard Sprague-Dawley (RjHan:SD) male breeders for OR and OP females, respectively. Six OP and six OR completed gestations and lactations after mating (**Figure 1A**).

Microbiota Sampling

Feces were collected 2 to 3 days before parturition (G3) and on days 5, 10, and 20 of lactation (**Figure 1A**). For vaginal and milk sampling, dams were anesthetized with isoflurane 2.5% + dioxygen (5 L/min) and shaved. Their ventral and anogenital area were carefully cleaned with 70% ethanol to ensure sterility. Vaginal smears were sampled around 2–3 days before parturition (G3) using a sterile swab (Puritan HydraFlock, Guilford, United States) humidified with sterile 0.9% NaCl and collected in a 2-ml sealed sterile tube (Sarstedt, Nümbrecht, Germany) containing 1 ml of 0.9% NaCl. Milk was collected as previously described (Martin Agnoux et al., 2015), at days 1, 5, and 10 of lactation (**Figure 1A**). Briefly, dams were injected intraperitoneally with 0.3 ml of oxytocin (Syntocinon, Sigma-Tau, Ivry-sur-Seine, France) (5 UI/ml) 15 min before anesthesia then milk was manually collected with sterile chirurgical gloves (Gammex Latex Ansell, Melaka, Malaysia) and sterile cottoned glass Pasteur pipette.

Inocula Preparation

Protocols for inocula preparation were chosen considering both feasibility and the need to mimic physiological conditions. To be close to the natural supply of milk-associated bacteria in one suckling [as calculated from our unpublished bacteria enumeration from rat milk and published data about milk consumption by rat pups (Bautista et al., 2008; Sevrin et al., 2017)], we had to deliver milk-derived inocula after bacterial cultivation. To mimic transmissions of fecal exchange between dams and pups, we inoculated 10^8 fecal bacteria per pup and per day.

Vaginal-,

fecal-,

and milk-derived inocula were prepared from the above microbiotal samples as detailed in **Supplementary Material and Methods** from OP and OR donors and were pooled 2 × 2 for each genotype in order to obtain enough material for carrying out microbiota transfer to offspring in duplicate experiments.

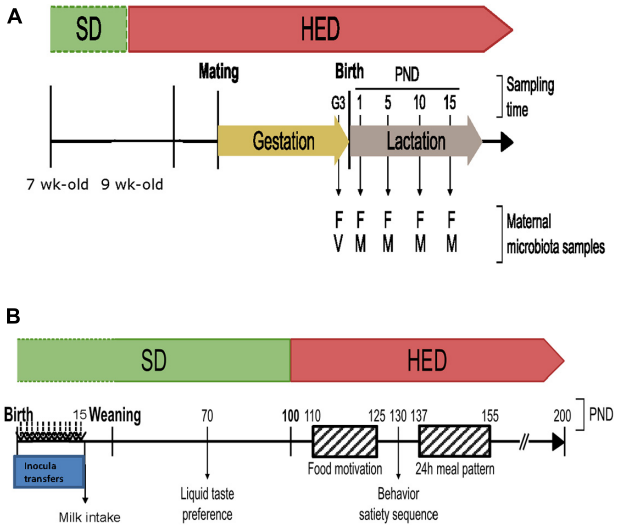

FIGURE 1 | (A) Timeline of maternal microbiota collection from OP and OR dams to produce inocula. Vaginal microbiota (V) was collected at the end of the third week of gestation (G3). Milk microbiota (M) was collected every 5 days from postnatal day (PND)1 to PND15. Fecal microbiota (F) was collected at every time point. **(B)** Inocula transfers and timeline of behavioral tests performed on Fischer pups before and after weaning.

Maternal Microbiota Transfer Experiment

Primiparous and conventional female Fischer (F344/HanZtmRj) rats (n = 18) were obtained at 1-day of gestation (G1) from Janvier-labs (Le Genest Saint Isle, France) and housed individually (22°C ± 2°C, 12:12-h reversed light/dark cycle) with free access to tap water and standard diet (A03, Safe Diet, Augy, France). This experimental protocol was carried on a total number of six litters for each experimental group during two independent sessions. The overall design of the study was as follows: for each dam, litter was culled to eight pups (four males and four females) that received as soon as possible the various inocula. For each session, three litters received the pools of inocula prepared from three pairs of OP dams (F-OP group), three litters received the pools of inocula prepared from three pairs of OR dams (F-OR group), and three litters received stock solution (F-Sham group). Pups' weight was monitored every day until postnatal day 15 (PND15) that corresponded to the end of microbiota transfers (**Figure 1B**). Pups were weaned at PND21 and housed individually (22°C ± 2°C, 12:12-h reversed light/dark cycle) with free access to tap water and standard diet

A03 (Safe Diet, Augy, France). They were then fed with the HED (58V8, TestDiet, Richmond, United States) from PND100 to the end of the experiment. Weight and food intake were regularly monitored from PND21 to PND200. Feces were harvested at PND60 and PND130.

All inocula were thawed at room temperature and gently transferred to pups by spontaneous oral intake using a micropipette. Vaginal inocula were transferred (5.0 to 5.7 log eq Bacteria per pup, **Supplementary Table 1**) first and only once within 2 h after birth (PND0) while both fecal and milk-derived inocula were administered daily, at the same time of day, from PND0 to PND15. For the latter, the volumes transferred were adapted as pups grew, resulting in a slight increase in the bacterial load (from 3.2 to 6.6 log bacteria per pup, **Supplementary Table 1**).

Cecocolonic and Cecal Contents

After death by intracardiac injection of 0.5 ml of Exagon® (Richter Pharma, Wels, Austria), the cecocolonic content of one male and one female pup was collected in each litter and pooled at PND11 ($n = 6$ per group). At PND20/21, cecocolonic contents were collected from three females/six males of the first session and six females/three males of the second one, in order to reach a number of nine rats per group and sex. Final numbers may differ from those expected due to sexing errors at birth. At PND20/21, the sizes of groups were as follows for females: F-Sham $n = 10$, F-OP $n = 8$, and F-OR $n = 9$; for males: F-Sham $n = 8$, F-OP $n = 10$, and F-OP $n = 9$. At adulthood, i.e., PND200/201 for the first session and PND196/197 for the second one, cecal contents were collected from three males/six females and six males/three females, respectively. Final effective were for females: F-Sham $n = 9$, F-OP $n = 9$, and F-OR $n = 7$; for males: F-Sham $n = 9$, F-OP $n = 9$, and F-OR $n = 11$. Contents were weighted and mixed in three times their volume in sterile water. After complete homogenization, these cecocolic/cecal suspensions were centrifuged (20 min, 7,800 × g, 4°C) then both supernatants and pellets were frozen at −20°C for microbiota analysis, respectively.

Microbiotal Characterization of Inocula and Intestinal Contents From F344 Recipients

DNA Extraction

DNA was extracted from inocula and from intestinal or fecal samples using kits from Qiagen, (Hilden, Germany) after enzymatic and possibly mechanical lysis according to protocols specifically adapted to the nature of the samples (see **Supplementary Material and Methods**).

DNA Quantification and Total Bacteria Count by qPCR

DNA concentrations in extracts were quantified using a NanoVue™ spectrophotometer (GE Healthcare, Little Chalfont, Bucks, United Kingdom).

Total bacteria counts were enumerated on DNA extracts using real-time quantitative polymerase chain reactions (qPCR). The analysis was performed using conditions described in Fanca-Berthon et al. (2010) with the following forward and reverse primers: 5′-WCCTACGGGWGGCAGCAGTS-3′; 5′-TTACCGCGGCTGCTGGCACR-3′.

16S rDNA Sequencing

The V4 hypervariable region of the 16S rDNA gene was amplified from the DNA extracts by PCR using composite primers: 5′-CTT TCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGC GGTAA-3′ and 5′-GGAGTTCAGACGTGTGCTCTTCCGATCT GGACTACHVGGGTWTCTAAT-3′ which were based on the primers adapted from Caporaso et al. (2011) (i.e., 515F and 806R).

The PCR mixture was composed of 65 μl of DNA diluted at 1 ng/μl and 35 μL of mix. Mix was composed of 18 μl of nuclease-free water, 10 μl of 10× buffer, 2 μl of each primer at 20 μM, 2 μl of dNTP at 10 mM, and 1 μl of MolTaq (MolZym, Plaisir, France) at 5 U/μl. DNA template was amplified according to the following thermal conditions: 1 min at 94°C, 28 cycles composed by 30 s at 94°C, 1 min at 56°C, 1 min at 72°C, and a final extension step at 72°C for 10 min. Ten microliters of each product was used to verify amplification by gel electrophoresis on a 1% agarose gels stained with GelRed (Interchim, Montluçon, France).

Paired-end sequencing was performed on a MiSeq System (Illumina, San Diego, CA, United States) with v3 reagents, producing 250 bp reads per end, according to manufacturer's instructions by the GeT-PlaGe platform (INRA-Toulouse, France). The 16S rDNA reads were demultiplexed by Illumina run software at GeT-PlaGe platform. Reads were then analyzed with the FROGS v3 pipeline (Escudie et al., 2018) in Galaxy environment[1] as described in **Supplementary Material and Methods**.

Eating Behavior Assessment

The behavior tests performed on F344 recipient rats are summarized in **Figure 1B** and detailed below.

Milk Intake

At PND15, milk intake was estimated by the weight-suckle-weight (WSW) method adapted from Sevrin et al. (2017). Pups were weighted (m1, g) and separated from their mother for 2 h to ensure gastric emptying. Before returning to their mother, urine excretion was stimulated by rubbing the urogenital area, and pups were weighted (m2, g) to estimate the hourly metabolic waste weight (m2 − m1)/2. After 1-h suckling, pups were weighted again (m3, g). Milk intake was estimated as the relative bodyweight gain for each pup (m3 − m1) + absolute value (m2 − m1)/2.

Liquid Taste Preference (Two-Bottle Choice Test)

Preference for sweet and fat tastes was measured at PND70 using the two-bottle choice test experiment as previously described (Paradis et al., 2017). After a 2-day period of adaptation to the presence of two bottles in their own cage, rats were free

[1] https://vm-galaxy-prod.toulouse.inra.fr/galaxy/

to choose between a bottle of tap water (control) and a bottle containing tap water with saccharin (0.01%, *w*/*v*) for sweet taste, or a bottle of tap water with xanthan gum 0.3% (control) and a bottle with corn oil (0.1%, *w*/*v*) and xanthan gum 0.3% for fat taste. Taste solutions provide no or negligible amount of calories to drive caloric satiety response (Paradis et al., 2017). Drink intake was measured daily for 3 days, and bottles were daily inverted to prevent position preference bias. The sweet and fat preference scores were calculated as the ratio between the volume of saccharin or fat solution consumed and the total drink intake in 24 h and then multiplied by 100. A preference for one of the drinks is defined above 50%.

Food Motivation

From PND110 to PND125, food motivation was assessed with the straight alley test as described by Pecina et al. (2003) and Wong et al. (2009). This was performed during the active phase, under red light using a 2-m-long black plastic device composed of a starting box (SB) (20 cm × 10 cm × 30 cm), a central alley (160 cm × 10 cm × 30 cm) and a goal box (GB) containing food reward (20 cm × 10 cm × 30 cm). Transparent movable Plexiglas doors separated the SB and the GB from the alley allowing choosing the setting of the GB along the alley. In order to choose the most palatable food reward, animals were habituated to the open-field area then tested in it with several palatable food [milk chocolate (Milka, Mondelez, France), chocolate cereals (Chocapic®, Nestlé, Switzerland), ham (Fleury Michon, France), almond paste (Maître Prunille, France), potato crisp (Original Pringles, Kelloggs, United States), and cheese (Leerdammer®, Bel, France)]. The number of nose contacts with each food was counted. Males demonstrated a preference for cheese and females for almond paste therefore for the following test, cheese (Leerdammer®, Bel, France) was used as food reward for males whereas almond paste (Maître Prunille®, France) was used for females.

Before the beginning of the test, a bit of cheese or almond paste was given for 3 days to male and female Fischer offspring, respectively, in their own cage, to extinguish food neophobia. During the training period, animals were fasted overnight (16–17 h) and trained in the afternoon. At D1, rats were placed one by one in the SB and after 30 s adaptation, Plexiglas door was removed and animals were free to go to the GB containing the reward pellet (5 g). Maximal duration of the test was 2 min. During the next training sessions, the distance between the SB door and GB was increased from 30 to 60, 100, 140, and 160 cm (maximum distance). For the test (D10), animals were fed *ad libitum* overnight in order to measure motivation rather than hunger. All trials were video-recorded and position, running, pausing, rearing, and turnaround behaviors, as well as latency to leave the SB, to reach GB and eat reward pellet were measured by a trained "blind" experimenter using BORIS® software (v.7.7.3) (Friard et al., 2016) (ethogram in **Supplementary Table 3**). An ingestion score was calculated from the latency to ingest the food reward with 1 point removed each 30 s (ingestion between 0 and 30 s = 3 points; between 31 and 60 s = 2 points; 61 and 90 s = 1 points; 91 and 120 s = 0 point). Since straight alley test is based on locomotor activity, a 5-min recorded open-field test was done the day after trial 10 to check locomotor activity or anxiety that showed no differences between rats.

Twenty-Four-Hour Eating Pattern

Between PND137 and PND155, eating behavior was analyzed in physiological cages (Phecomb cages, Bioseb, Vitrol, France), as previously described (Coupe et al., 2011; Le Drean et al., 2019). Briefly, rats were allowed to adapt individually to physiological cage during 1 day before data recording. Data were recorded from the beginning of the second day (8:00 a.m.) each 5 s over a 24-h period. Meal was defined as 0.1 g food intake during a minimum of 10 s with an intermeal period of 10 min minimum. Meal parameters extracted from Compulse software (v.1.1.01) (PheCOMP, Panlab, Spain) included latency to eat, number of meals, meal size, and duration of intermeal interval and satiety ratio. A percentage of reliability of the quality data was calculated by the software, and only behavioral items reaching a percentage of reliability >80% have been used.

Behavioral Satiety Sequence Analysis

At PND130, the analysis of the behavioral satiety sequence (BSS) was performed as described (Halford et al., 1998) in animal home cage. This was done by videotaped, under red light, animals that received 90 g HED (58V8, TestDiet, Richmond, United States) in their home cage for a 90-min period after an overnight fast. Total food intake was measured, and activities as feeding, grooming, resting were quantified. The test period was divided into eighteen 5-min time bins, which allowed determining meal duration, feeding rate, grooming, and resting time, as well as the time of occurrence of satiety. One female F-Sham and one male F-OP were removed for demonstrating an abnormal lethargy.

Behavioral *Z*-Scoring

In order to reduce the intrinsic variability of single tests, we integrated complementary and convergent measures by *z*-normalization along a same behavioral dimension (propensity to eat, preference for more sweet or fat, see **Supplementary Table 4**), as previously described (Guilloux et al., 2011). This *z*-normalization was calculated according to group and sex.

Statistical Analysis

All univariate statistical analyses were performed with GraphPad PRISM® software (v.6.01). Because of a low number ($n < 8$) of weaned female OR, we could not check for normality, and because of large differences in variance between groups, we applied non-parametric tests to all datasets, unless otherwise stated. All parameters of growth, food consumption, and eating behavior were analyzed using Kruskal–Wallis analysis of variance and comparison between groups using *post hoc* Dunn's test. Wilcoxon signed rank test was used to compare group values to a reference value [calorie intake under SD during the first 10 days on HED, no preference–no aversion (50%) during liquid preference test and zero for behavior *z*-scoring].

Linear discriminant analysis effect size (LEfSe) (Segata et al., 2011) was used on the online Galaxy interface (see Text Footnote 1) to investigate bacterial markers that drive differences between groups. Other multivariate statistical analyses were performed under R® (v.3.6.1.) with the FactoMineR® 2.2 Package (Le et al., 2008). Beta diversity was analyzed through clustering of dissimilarity measures (Cao) using the ggdendro® (v.0.1-20) and phyloseq® (v.1.28.0) packages (McMurdie and Holmes, 2013). Relations between eating behavior characteristics and microbiotal features were searched using unfold principal component analysis (UPCA) and by calculation of Spearman correlation coefficients and their level of significance. Using a classical principal component analysis, UPCA helps to link the most relevant behavioral characteristics to the most relevant bacterial abundances, in a simplified subspace of the initial datasets. When these analyses were done on data arising from different ages (i.e., different animals, as, for example, when associating microbiotal features at PND21 and eating behavior characteristics at PND200), the pair fitting was made on the basis of sex and litters belonging.

RESULTS

Inocula From OP and OR Dams Differed

As expected, fecal inocula exhibited higher OTU richness than milk-derived ones (**Supplementary Table 5**), and their bacterial composition were very different (**Figure 2A** for the family level). Milk-derived inocula predominantly consisted of *Enterobacteriaceae* (ca. 28%), *Staphylococcaceae* (ca. 23%), *Streptococcaceae* (ca. 17%), and *Pasteurellaceae* (ca. 17%) while fecal inocula composition was dominated by *Lachnospiraceae* (ca. 35%) and *Ruminococcaceae* (ca. 15%).

Within each inoculum sampling site and taking into account all sampling times, shared OTUs constituted the major fraction (79.2 and 48.7% for feces- and milk-derived inocula, respectively), but differences according to the genetic background were observed. These were found to be insignificant on alpha-diversity indexes (**Supplementary Table 5**), but clear-cut separations were evidenced by clustering of dissimilarity measures (Cao) between samples (**Figure 2B**). We also identified various taxa with significantly differential abundances according to the genetic background using LEfSe analysis (**Figure 2C**). These were numerous for fecal inocula with the highest LDA scores being observed for members of *Clostridiaceae 1*, *Tannerellaceae*, and *Erysipelotrichaceae* (more prevalent in OR), as well as members of *Desulfovibrionaceae*, *Parabacteroides*, or *Anaerostipes* (more prevalent in OP, **Supplementary Figure 1A**). Conversely, milk-derived inocula from OP were discriminated from those originating from OR by only four genera and one species: *Streptococcus* sp. and two unclassified genera affiliated to *Pasteurellaceae* or *Staphylococcaceae* were more prevalent in OR while one unclassified genus among *Carnobacteriaceae* and one unclassified species from *Staphylococcus* sp. were more prevalent in OR (**Figure 2C** and **Supplementary Figure 1B**).

Characterization of vaginal inocula did not succeed due to extremely low concentrations of bacterial DNA (1.0 ± 0.4 ng/µl), and their composition based on 16S sequencing was equivalent to that observed in the negative controls (data not shown).

Inocula Transfer Induced Transient Changes in Gut Microbiota of Recipients

Inocula transfers modified the composition of intestinal microbiota in F344 recipients before weaning (**Figures 3A–C**) without significantly impacting the absolute amounts of bacteria (**Supplementary Table 6**). Microbiotal changes were mainly observed at PND21, i.e., 6 days after the last transfers. Indeed, at PND11, cecocolonic bacterial communities of F-sham, F-OP, F-OR, and F344 recipients did not significantly differ in terms of alpha- and beta-diversities nor dominant families (**Figures 3A–C**). However, following LEfSe analysis (**Figure 3C**), gut microbiota from both the F-OP and F-OR groups stood out significantly from the F-Sham group by their contents in bacteria of *Family XIII* (LDA score = 2.47) and in an unknown species affiliated to the GCA-900066225 group in *Ruminococcaceae* (LDA score = 2.06) (**Supplementary Figure 2**).

Conversely, at PND21, observed richness was increased while alpha diversity, as estimated by Simpson's reciprocal index (i.e., 1/D), was decreased in females and males from the F-OP group as compared with their F-Sham counterparts (**Figure 3A**). In addition, in a very obvious way for males and to a lesser extent for females, clear differences between intestinal microbial communities were demonstrated by hierarchical clustering of dissimilarity values (Cao) between microbiotas from the F-OP and F-OR groups—which did not separate from each other—and those from the F-Sham group (**Figure 3B**). Accordingly, many taxa were identified by the LEfSe analysis as characteristic of both the F-OP and F-OR groups, when compared with the F-Sham group (16 for females and five for males, **Figure 3C**). However, this analysis also allowed the identification of taxa specific of either the F-OP or F-OR group (six and 19 for females and males from the F-OP group and 18 and six for females and males from the F-OR group). These taxa belong to both ultradominant and subdominant families (e.g., *Lachnospiraceae* and *Atopobiaceae* in the case of the F-Sham females) (**Supplementary Figure 3A**). Depending on the group, the nature of these taxa drastically differed or not for each sex. For F-OR, only an unclassified species belonging to the *Holdemania* sp. constituted a marker found in both males and females. Conversely, in the case of F-OP, the five taxa identified as characteristic in males were also found in females with LDA scores of the same order of magnitude (see **Supplementary Figures 3A,B**).

After weaning, in growing and adult animals, analyses led to heterogeneous results suggesting that microbiotal changes, if any, were of small magnitude. Using the LEfSe analysis, a few taxa were identified as characteristic of the different groups, at most sampling times for both sexes, but no overall picture emerged from the time course of their numbers as the animals aged (**Supplementary Figures 4A–D**). Within a same group, most of these bacterial markers differed according to

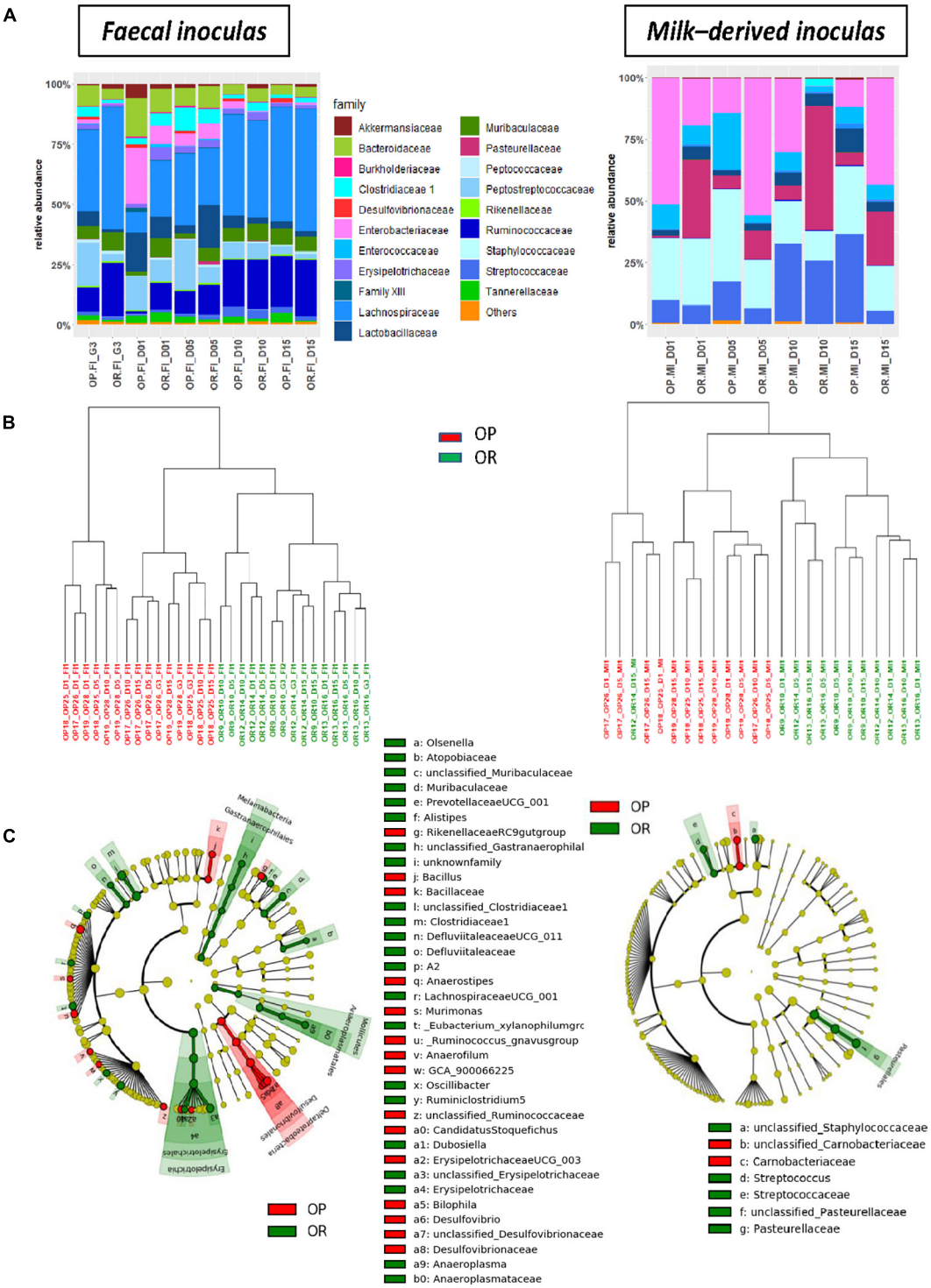

FIGURE 2 | Bacterial composition and structure of inocula prepared from OP and OR dams (n = 3 per genetic origin and sampling time). **(A)** Relative abundances of bacterial family and **(B)** Cluster dendrograms of dissimilarities measures (Cao) in inocula according to sampling time (OP-inocula are in red and OR-inocula are in green). **(C)** Taxonomic cladograms obtained from LEfse analysis of OTU abundances, showing as circles (which diameters are proportional to abundances), taxa that significantly ($p < 0.01$) discriminate inocula according to their genetic origins, on concentric rings from domain in the innermost one to genera in the outermost (see **Supplementary Figure 1** for LDA values).

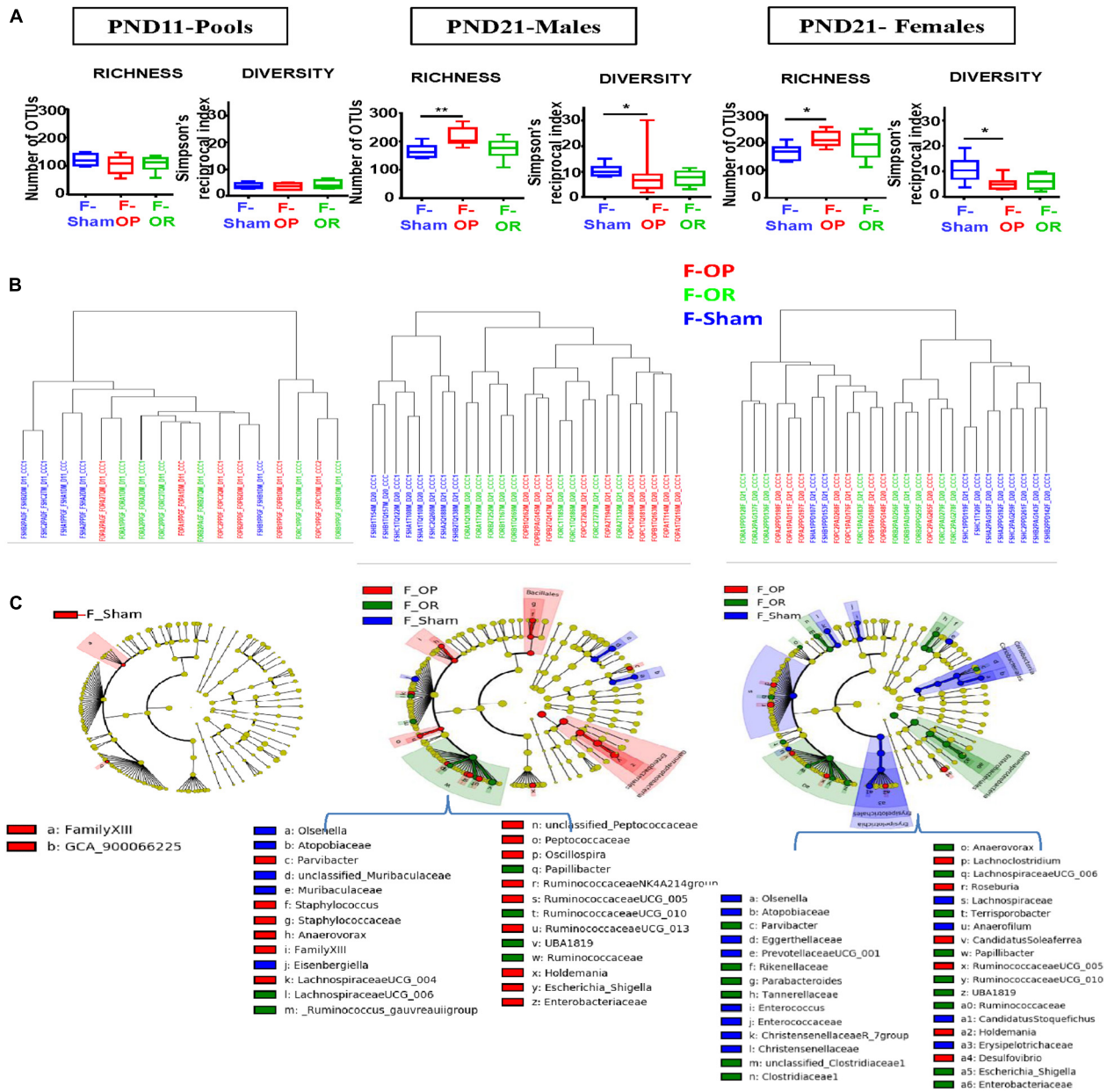

FIGURE 3 | Structure and composition of cecocolonic microbiotas of F344 recipients before weaning (n = 5 to 10 samples per group and per sex). **(A)** Observed richness and diversity. **(B)** Cluster dendrograms of dissimilarity measures (Cao) between individual samples (F-Sham are in blue, F-OP in red, and F-OR in green). **(C)** Taxonomic cladograms obtained from LEfse analysis of OTU abundances, showing as circles (which diameters are proportional to abundances), taxa that significantly ($p < 0.05$) discriminate samples according to the experimental group, on concentric rings from phyla in the innermost one to species in the outermost, see specific color legend for each sampling time (see **Supplementary Figure 3** for LDA values).

sex or age. The few exceptions were (i) an unknown species from *Candidatus Soleaferrea* sp. which was associated to the F-OP group at both PND130 and PND200 in males and (ii) the genera *Ruminiclostridium* and *Enterorhabdus* which were associated to the F-OR group in both males and females. In addition to a larger number of associated taxa, the F-OR group was also distinguished by significant differences in observed richness, which was increased in females from the F-OR group at PND60 (257 [226–282] as median and [range]) and PND130 (299 [281–310] as compared with their counterparts from the F-Sham group (224 [184–244] and 272 [241–285], respectively). Conversely, experimental groups could not be distinguished anymore by the beta-diversities of the microbiotal communities (**Supplementary Figure 5**).

When compiling all the bacterial species identified as discriminative of the different types of microbiota (**Figure 4**), it

appears that very few of those identified from the inocula were retrieved in the F344 recipients. Therefore, the modification of the F344 recipients' microbiota by transfer did not identically replicate the transferred inocula.

Neonatal Transfer of Maternal Microbiota Had Little Effect on Early Growth but Impacted Feeding Behavior

Pup Growth Before Weaning

Pup bodyweight was monitored daily from birth until PND15 (**Figures 5A,B**). Except for one transient difference at PND1 and a trend at PND2, no significant difference was observed between the F-OR, F-Sham, and F-OP groups. Both female (**Figure 5A**) and male pups (**Figure 5B**) from the F-OP group weighed less than their counterparts from the F-Sham group. This difference reached statistical significance from PND9 until PND15 for females and through the entire period for males. Nevertheless, no significant difference was observed for body weight gain measured at PND22 (only on fully weaned pups) (**Figures 5C,D**).

Milk Intake

Hourly milk intake (as estimated by relative weight gain after 1-h suckling) at PND15 was significantly lower for both male and female F-OR than F-OP pups and lower for F-OR males as compared with F-Sham (**Figures 6A,B**).

Pups' Growth After Weaning

From PND25 to PND78, energy intake from SD was higher for F-OP females than F-Sham females (**Figures 6C,D**) but without any effect on weight gain (**Figure 6E**). During the same period, no significant difference between groups of males was observed (data not shown).

Rats were then switched to HED from PND100 to the end of the experiment (PND200), and food consumption was recorded daily from PND99 to PND109. Their energy intake rose to stabilize later and differently between females and males. For females, no difference in energy intake was detected between groups during the first 10 days of HED (**Figure 6F**) nor in body weight gain (**Figure 6H**). F-OP females adjusted their food intake in 3 days, when F-Sham and F-OR adjusted their food intake in 7 days (**Figure 6F**). Concerning males, despite no difference in caloric ingestion between groups during the first 10 days of HED (**Figure 6G**), F-OR gained significantly less weight than F-OP rats (**Figure 6I**). F-Sham and F-OR males adjusted their food intake in 7 days, when F-OP males needed 8 days (**Figure 6G**).

During the behavioral tests from PND110 to PND155, no significant difference in body weight, body weight gain, or food intake was noticed between groups among female and male F344 recipients.

Liquid Taste Preference (Two-Bottle Choice Test)

From PND75 to PND85, taste preference was measured using the *two-bottle choice test*. All animals significantly preferred sweet- and fat-tasting solutions, expressed as a percentage of consumption of taste solution above 50%, and no difference was observed between groups (**Figures 7A,B**). However, the total intake of sweet solution during the 3-day test tended to be lower in F-OR females (**Figure 7C**) and the total intake of fat solution was significantly lower in F-OR males (**Figure 7D**).

Food Motivation

From PND110 to PND125, food motivation was assessed by the *straight alley test*. On the test day, both F-OP and F-OR females and males seemed to be more focused than the F-Sham groups on their way to reach the GB, with less stops and rearing, but this effect was not significant (**Figures 8A,B**). Both male and female F-OP showed a higher frequency of eating the reward (score 3) compared with F-Sham and, to a lesser extent, F-OR (**Figure 8B**). The greater performance in the F-OP groups was revealed by a Chi^2 test showing significantly more success in eating the reward, for females and males (**Figure 8C**). These differences did not depend on the overall activity (locomotion or stops) during the first path to the palatable reward (GB) (data not shown) or a state of anxiety that was evaluated 1 day after the straight alley test in the open-field test (data not shown).

Twenty-Four-Hour Eating Pattern

From PND137 to PND155, meal pattern was characterized in detail over a 24-h period. No significant difference in total food intake was noticed between groups. During the 12-h diurnal phase, F344 recipient rats consumed a negligible amount of food that did not differ between groups (data not shown); we therefore focused on the nocturnal phase. No difference was revealed concerning the first nocturnal meal of the females (**Figures 9A,B**). Male F-OR consumed their first nocturnal meal significantly quicker as compared with male F-OP (**Figure 9A**), and the meal was bigger for male F-OP than male F-Sham (**Figure 9B**). During the 12-h dark period, in female F-OR, the number of meals was smaller as compared with F-Sham (**Figure 9C**), with a trend toward a higher ingestion speed per meal (**Figure 9D**), but mean food intake per meal did not significantly differ (**Figure 9E**).

Behavioral Satiety Sequence

Behavioral satiety sequence was monitored over a 90-min period at PND130. Females and males from the F-Sham group exhibited a typical BSS characterized by an initial eating period of approximately 30 min followed by grooming, then sleeping (**Figure 10A**). In females, the satiety state occurred after 40 and 55 min for F-OR and F-OP, respectively. The longer eating period featured by F-OP than F-Sham females resulted in a significant increase in the "activity/sleep" and "eat/sleep" ratios (**Figures 10B,C**).

In males of the F-Sham group, the sleeping period started 25 min postrefeeding (**Figure 10A**), around 35 min for the F-OR group and 45 min for the F-OP, but due to the lack of clearcut difference between behaviors, no significant difference was observed in activity/sleep and eat/sleep ratios between male groups (data not shown).

Integrated Behavioral *Z*-Score

For F-OP females, the integrated behavioral *z*-score was significantly greater than zero, which means for this group an increased risk of eating behavior which favors higher food consumption, a preference for fat or sugar, and higher motivation

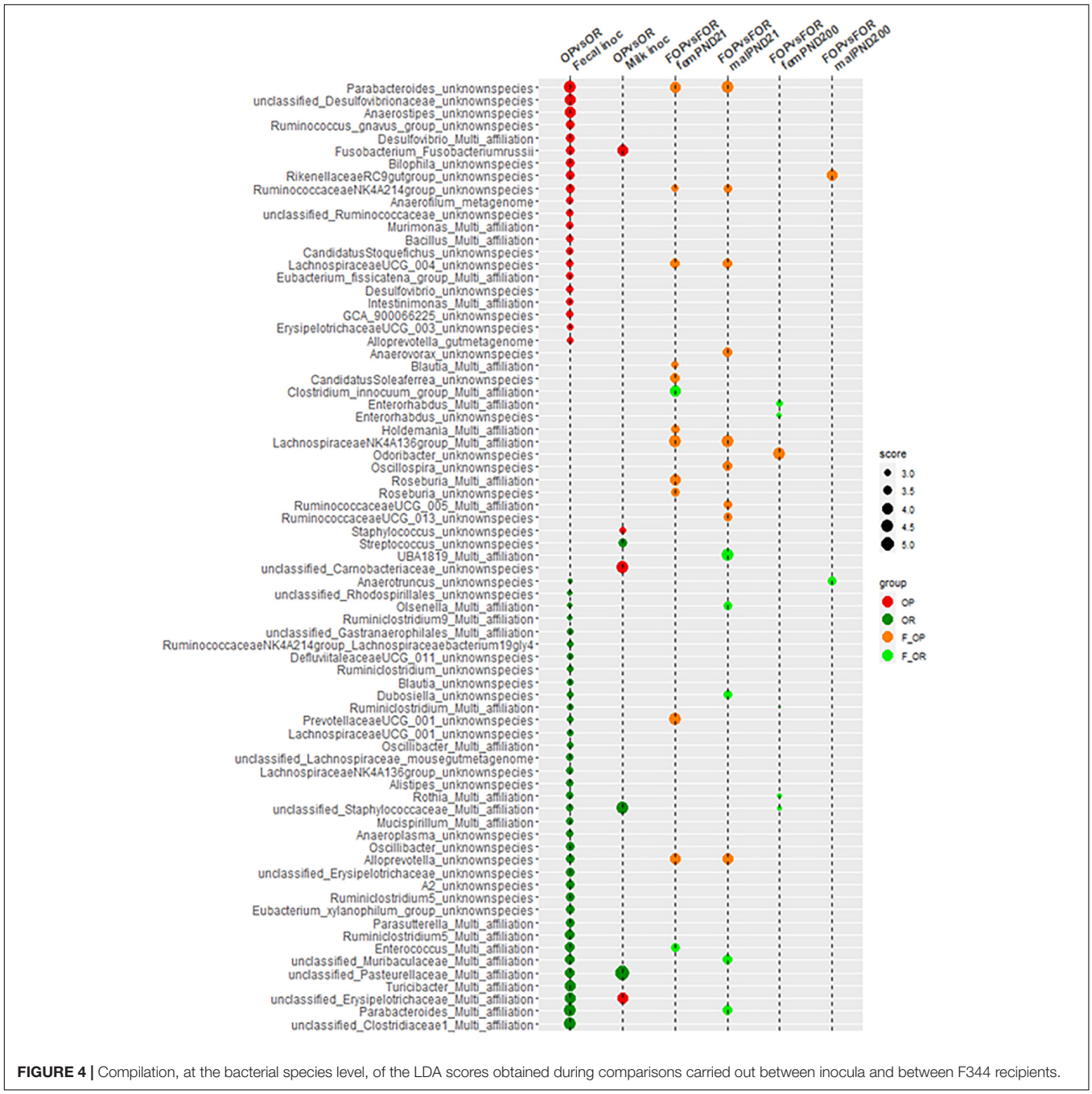

FIGURE 4 | Compilation, at the bacterial species level, of the LDA scores obtained during comparisons carried out between inocula and between F344 recipients.

for food (**Figure 11**). For males of the F-OP group, the integrated behavioral *z*-score was significantly higher than that of their F-OR counterparts, conferring to the former a riskier eating behavior.

Behavioral Characteristics of Adults Were Associated With Both Juvenile and Adult Microbiotal Traits

To determine whether the feeding behavior in adult recipient rats could be associated with specific characteristics of the microbiota, we performed an unfold PCA analysis which is a simple and straightforward method of analysis to investigate the relationships between two datasets after pair fitting made on the basis of sex and litters belonging when necessary. As a few data are lacking for some animals regarding behavioral tests, the analysis could not be applied to all individuals.

When considering microbiotal data at PND21, for males and females, no specific relationship could be detected between eating behavior characteristics and bacterial family abundances, which both contribute mostly to the first two components (**Supplementary Figure 6A**). Conversely, for OTU abundances

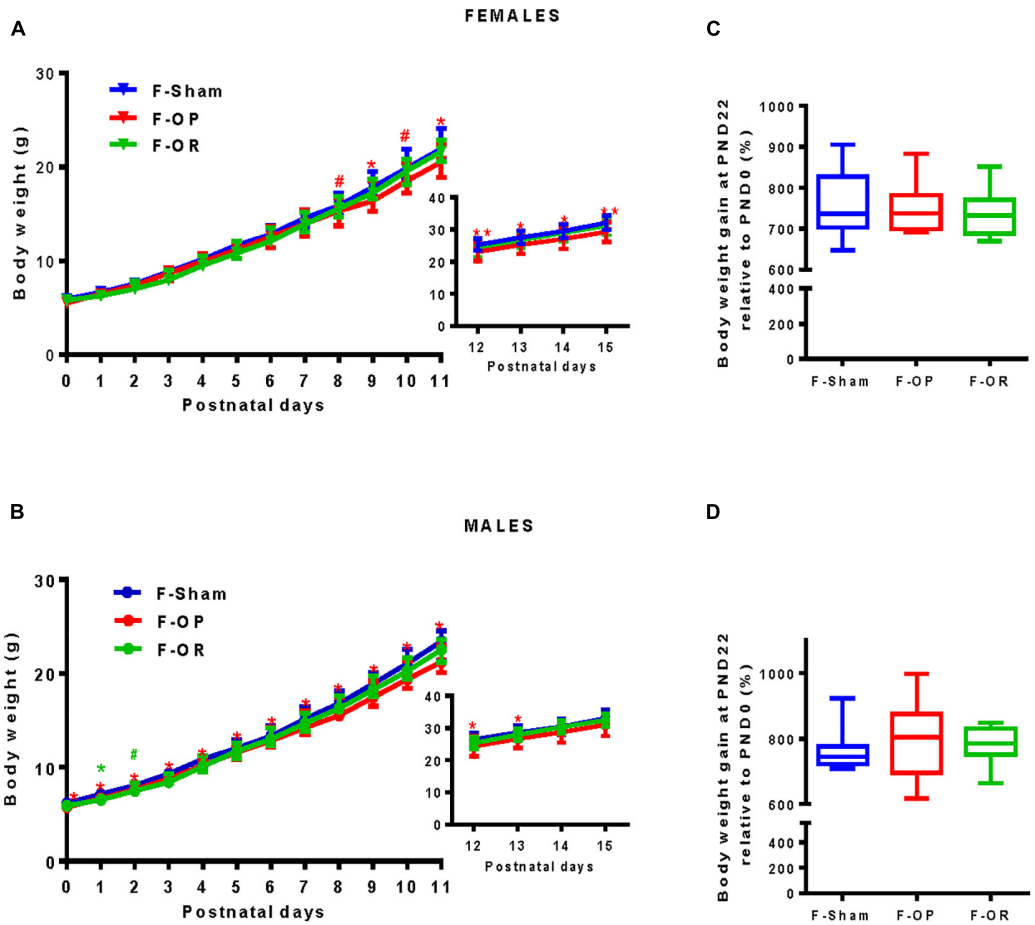

FIGURE 5 | Pups' growth during the lactation period. Bodyweight of female **(A)** and male **(B)**, from birth to PND11 (females n = 22–25, males n = 23–26) and in inserts from PND12 to PND15 (females n = 16–19, males n = 17–20), data are medians and interquartile range, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.1$, Kruskal–Wallis and *post hoc* Dunn's test at each PND. "*" and "**" indicate differences ("#" tendency) between F-OP and F-Sham; "*" indicates differences ("#" tendency) between F-OR and F-Sham. **(C,D)** Relative bodyweight (%) obtained by dividing body weight gain at PND22 by body weight at birth of females (**C**, n = 7–9) and males (**D**, n = 9–11).

(**Supplementary Figure 6B**), both "total cumulated SD consumption" and "SD efficiency" appeared related to the abundance of one specific member of *Lactobacillus* sp. (cluster 470) and of one OTU affiliated to *Ruminococcaceae* NK4A214 group (cluster 251) in females while the "average duration of sleep" was related to the abundance of one unclassified *Lachnospiraceae* (cluster 93) in males. All these associations were observed while UCPA allowed a good separation between individuals from the Sham groups and those from the FOP and FOR groups (data not shown) and therefore may have been induced by treatments.

Regarding adult microbiota abundances, associations were only found for females for which both "blundell_eat.to.sleep_min" and "HED efficiency during the first week" were aggregated with abundances of three specific OTUs, belonging to unclassified *Lachnospiraceae* (cluster 134), *Oscillobacter* sp. (cluster 343) and *Ruminiclostridium 6* (cluster 449), respectively (**Supplementary Figure 7**).

To further investigate associations between microbiotal and behavioral characteristics, a search for Spearman correlation between individual variables was carried out after selecting behavioral variables representative of the functions affected by treatment. Interestingly, significant correlations were found not only for bacterial abundances measured at adulthood, i.e., at the same stage of life as behavior testing but also for those determined before weaning (**Figure 12**). In both cases, the identified taxa belonged to a large range of bacterial families, but this was exacerbated when using preweaning microbiotal data. None of the correlated couples that were identified using the PND21 data was recovered when using 16S data from adults. The sole exception was a correlation between members of *Ruminococcaceae* and the integrated behavioral z-score in females; however, the precise nature of these members differed, while the direction of the correlation was inverted.

For females, the strongest associations (**Supplementary Figure 8A**) corresponded to (i) an inverse relationship between the "satiety ratio after the first night meal" and the abundance of the *Clostridium innocuum* group or one of its constituent clusters (cluster 102) at PND21, (ii) an inverse relationship between the "cumulated consumption of SD" and the abundance of the

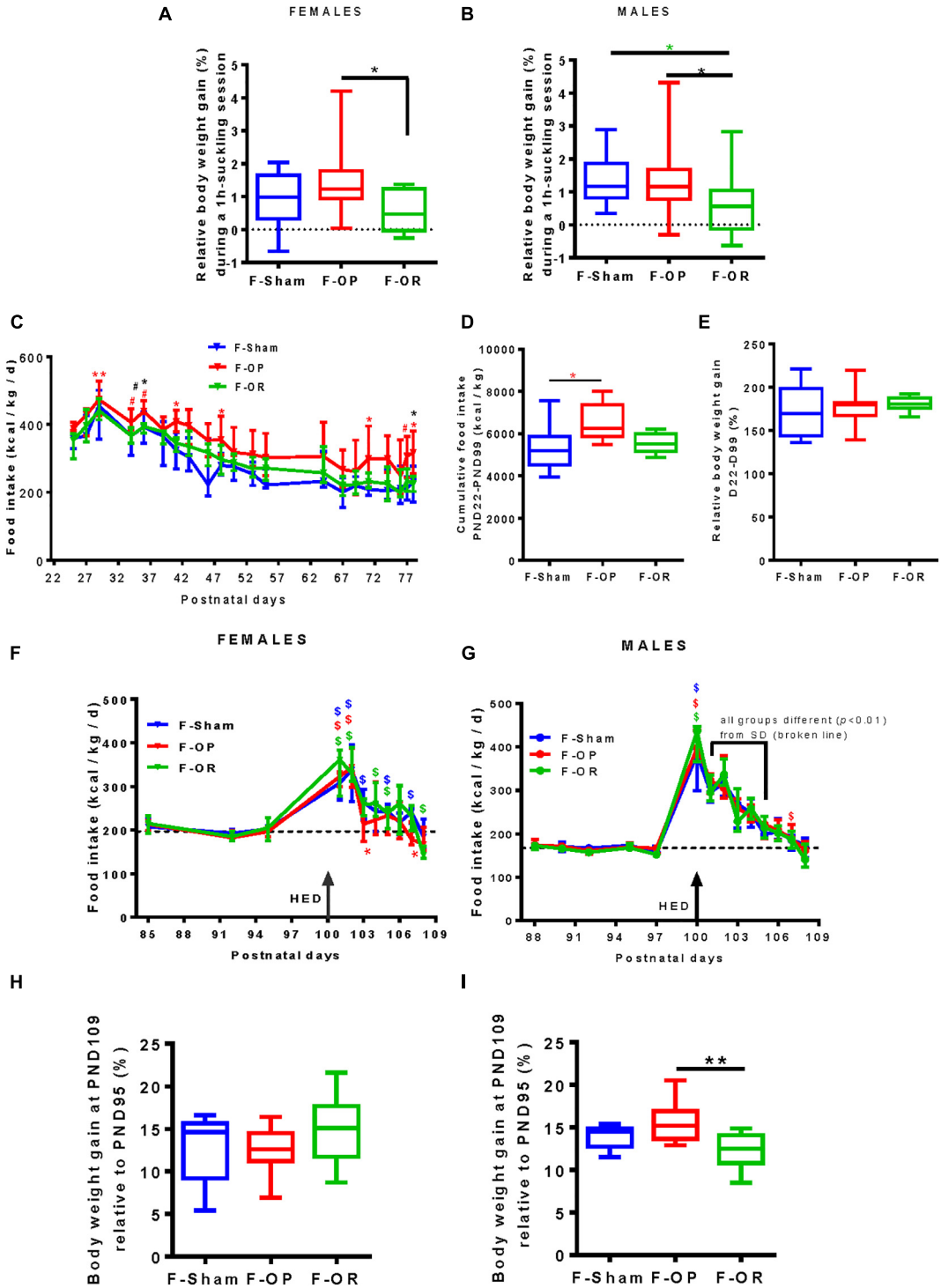

FIGURE 6 | Food intake at different periods of life. Hourly milk intake of female **(A)** (n = 16–19) and **(B)** male pups (n = 17–20) at PND15 estimated by the body weight gain after suckling divided by initial body weight. Daily **(C)** and cumulative **(D)** food intake and **(E)** relative weight gain on SD after weaning in females (n = 7–9). **(F,G)** Daily caloric intake and **(H,I)** relative weight gain during the first 10 days of HED (PND100–109) in females (left, n = 7–8) and males (right, n = 9–11). Panels **(A,B,D,E,H,I)** data are medians with range (min–max), $^{*}p < 0.05$, $^{**}p < 0.01$, Kruskal–Wallis and *post hoc* Dunn's tests. **(C,F,G)** Data are medians and interquartile range, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}0.05 < p < 0.06$; "*" indicates differences ("#" tendency) between F-OP and F-Sham; "*" indicates differences ("#" tendency) between F-OP and F-OR. **(F,G)** $^{\$}$, $^{\$}$, $^{\$}p < 0.05$ indicate difference from basal SD consumption (dotted line, median of the three groups) in each group, Wilcoxon signed rank test.

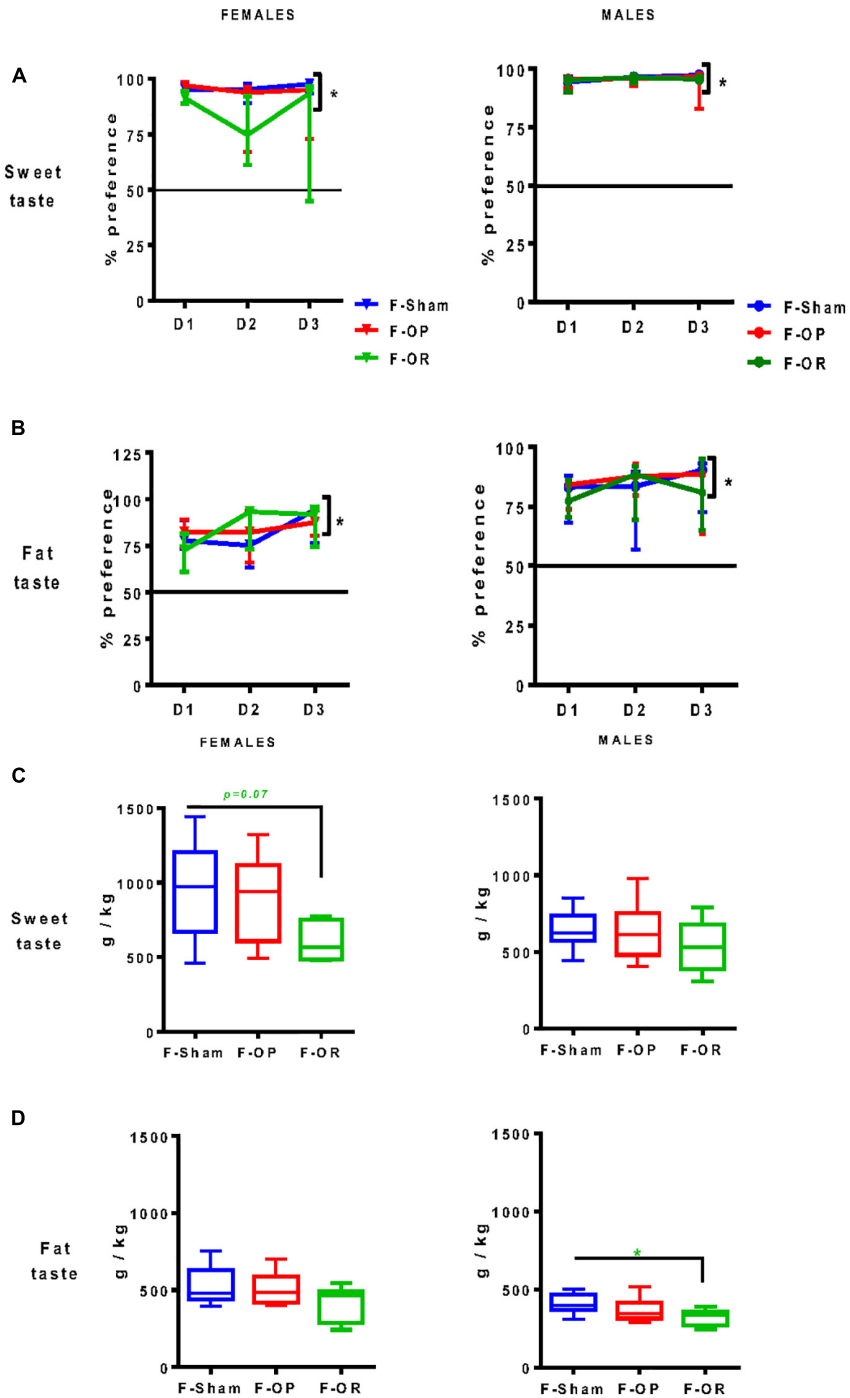

FIGURE 7 | Liquid taste preference. Preference for sweet **(A)** and fat **(B)** tastes and total intake of liquids drunk during the 3-day test for sweet **(C)** and fat **(D)** tastes in females (left, n = 7–9) and males (right, n = 8–11). Data are expressed as medians and interquartile range. **(A,B)** *$p < 0.05$, indicates difference from 50%, Wilcoxon signed rank test. Differences between groups were characterized by Kruskal–Wallis and *post hoc* Dunn's at each day of the test. **(C,D)** *$p < 0.05$, Kruskal–Wallis and *post hoc* Dunn's test. Animals were 75–85-day old and fed SD.

family *Pasteurellaceae* or two of its components (an unclassified genus and cluster 8), and (iii) a positive relationship between "the rate of ingestion per night meal" and the abundance of the *Streptococcaceae* family or two of its components (*Lactococcus* sp. and cluster 20) in adulthood. For males, there were three inverse relationships, the first between "body weight gain under HED" and the abundance, at PND21, of cluster 77, affiliated to an unidentified genus of *Muribaculaceae*, the second between

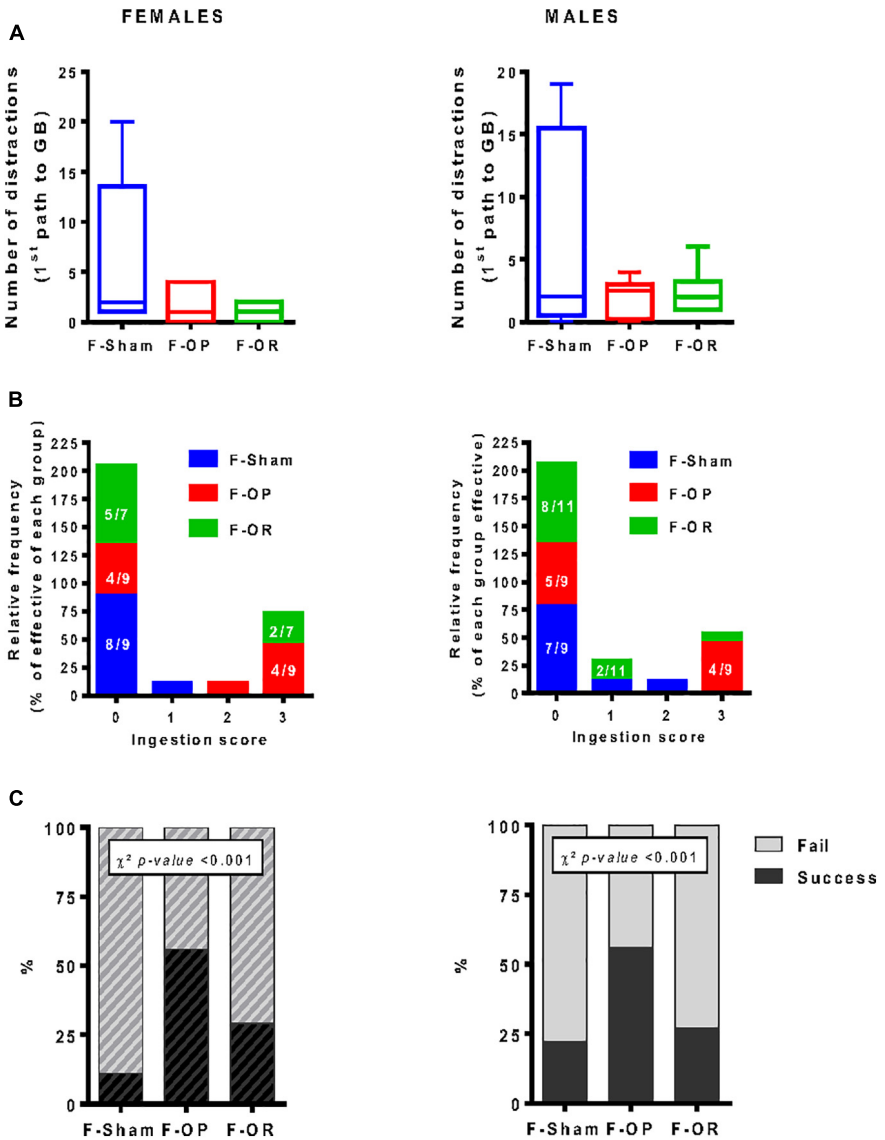

FIGURE 8 | Food motivation (straight alley test). **(A)** Number of distractions (stops and rearing) during the first walk to food reward, data are medians and min–max, Kruskal–Wallis test. **(B)** ingestion score (velocity to eat the food reward, scale 0 (lower score) to 3 (higher score), data are number values for each score (frequency). **(C)** Percentage of test success (eat reward)/failure (not eat reward), uniformity χ^2 test. **(A–C)** Left: females (n = 7–9), right: males (n = 9–11). Animals were 110–125-day old and fed HED.

"HED intake during the first week" and the abundance of cluster 2, affiliated to *Lactobacillus* sp. and the third between the satiety ratio after the first night meal and the abundance of *Bacteroides* sp., at adulthood (**Supplementary Figure 8B**).

DISCUSSION

To the best of our knowledge, the current report is the first to demonstrate that the transfer of maternal microbiota from mother to child can program the eating behavior of the offspring in a rodent model of obesity.

We chose to perform a vertical transfer of microbiota of dams that were genetically predisposed to obesity or not, both fed a high-energy diet, to newborn pups born from a control strain, unchallenged Fisher dams, so as to get rid of the genetic determinants of offspring obesity and to minimize confounding factors such as metabolic and the low-grade inflammation due to both obesity *per se* and the high-energy diet during gestation and lactation, in an effort to explore the sole impact of the transfer of different microbiota *per se* on feeding behavior phenotype. Our second objective was to identify whether the differences in the composition of early microbiota were long lasting and were associated with specific characteristics of feeding behavior.

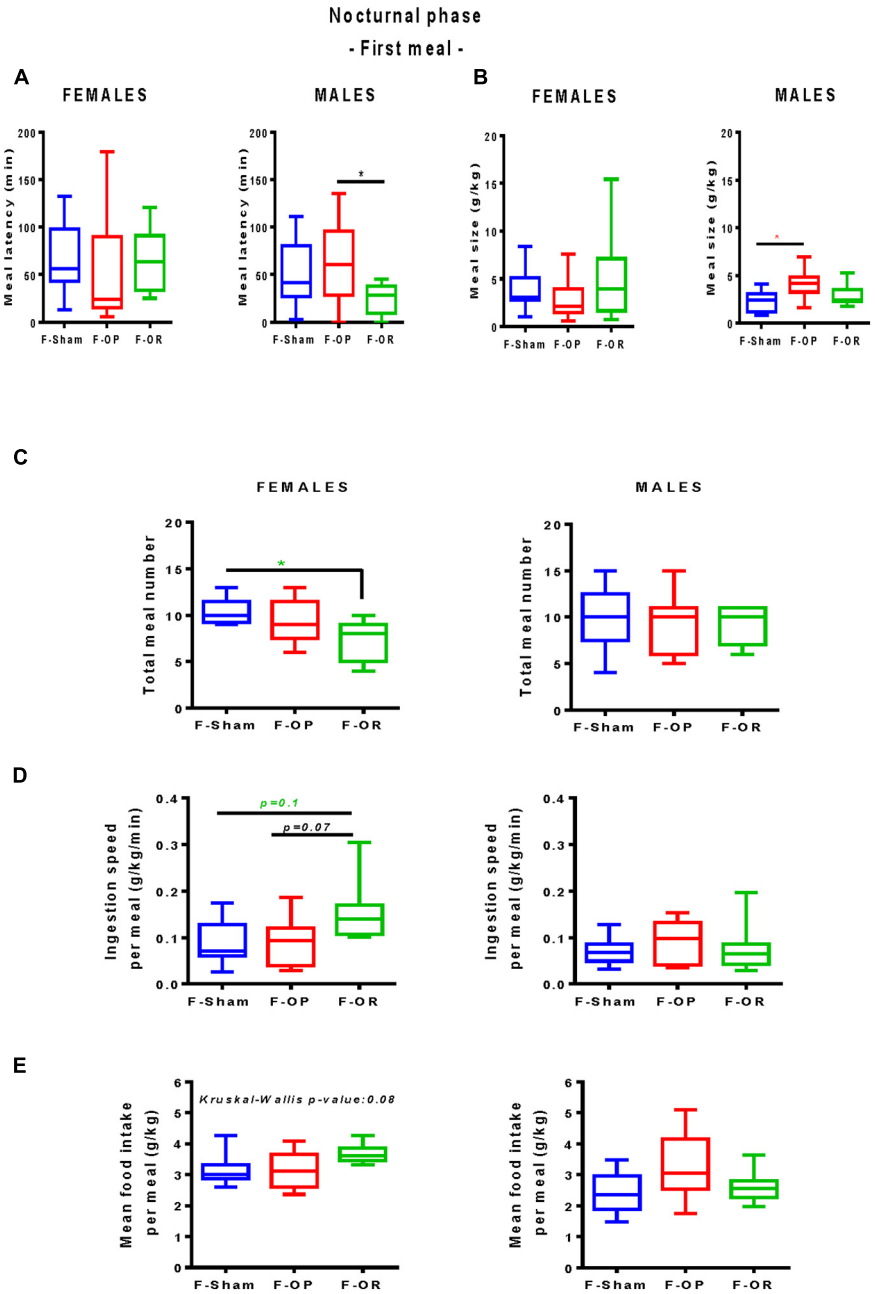

FIGURE 9 | Meal pattern. **(A)** Latency to eat and **(B)** meal size during the first nocturnal meal. **(C)** Total meal number. **(D)** Ingestion speed per meal and **(E)** mean food intake per meal during the nocturnal phase (12 h). Left: females (n = 7–9), right: males (n = 9–11). Data are medians and min–max values; *$p < 0.05$ indicate difference between groups, Kruskal–Wallis and *post hoc* Dunn's test. Animals were 137–155-day old and fed HED.

The Inocula Did Exhibit Different Bacterial Composition but Exerted a Transient Impact on Recipients' Intestinal Microbiota

The underlying assumption for our experimental design was that the microbiota obtained from several sites differed between OP and OR dams. Scattered studies conducted in either standard Sprague-Dawley or Wistar male rats with differential responsiveness to high-energy diets, or in the Sprague-Dawley OP and OR, which were initially selected (Levin et al., 1997) to respond differently to high-fat diets, support such assumption. Differences in the composition of intestinal or fecal microbiota were reported (de La Serre et al., 2010; Cluny et al., 2015), and Obanda et al. (2018) described a difference in the metabolic capacity of the microbiota, which has since also been associated

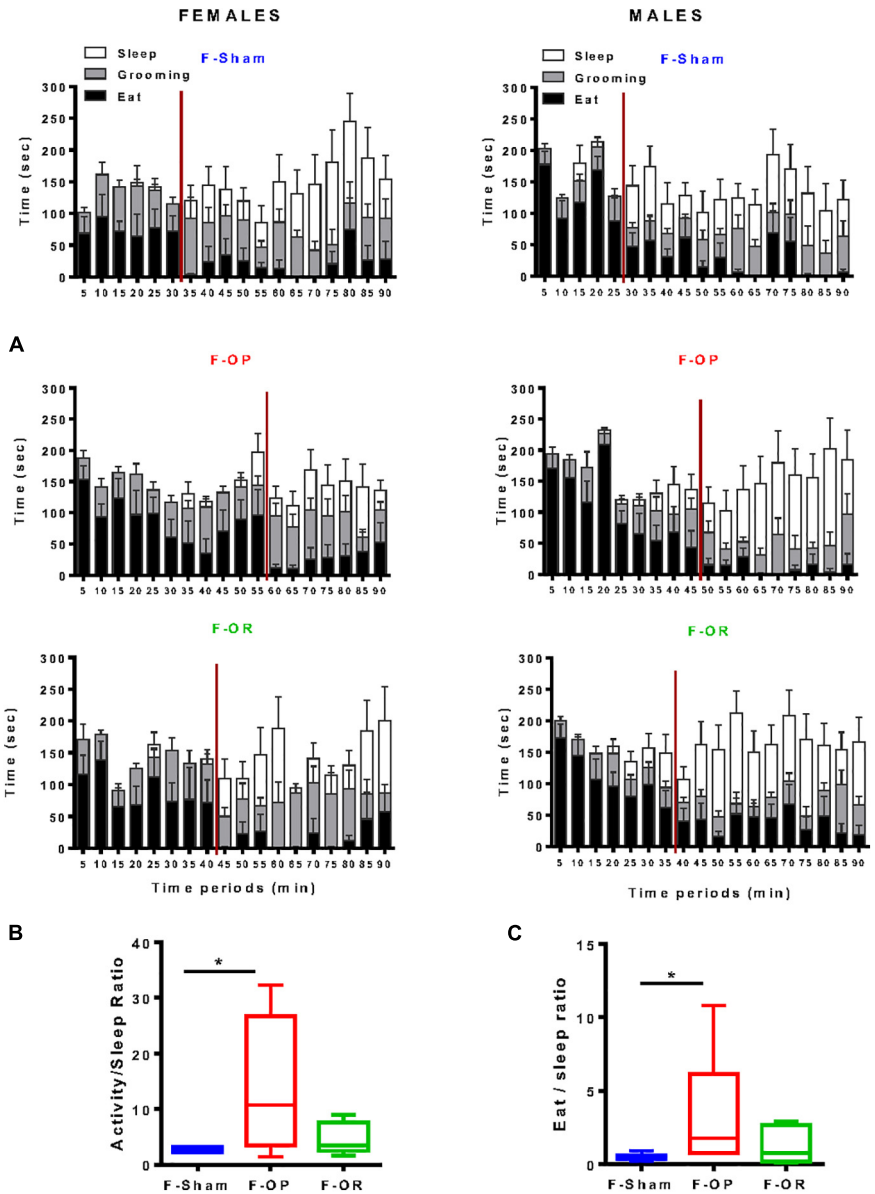

FIGURE 10 | Behavioral satiety sequence. **(A)** Blundell representations of behavioral satiety sequences for the three groups of females (left, n = 7–9) and males (right, n = 8–11), data are means ± SEM, satiety point is represented by the red vertical line and correspond to latency before behavioral switch between eat and sleep. **(B)** Activity-to-sleep ratio and **(C)** eat-to-sleep ratio in females (n = 7–9); data are medians and min–max values, *$p < 0.05$ indicates difference between groups, Kruskal–Wallis and *post hoc* Dunn's test. Animals were 130-day old and fed HED.

with changes in the cecal abundance of some specific strains (Obanda et al., 2020).

Despite the known impact of gender on microbiota composition (e.g., Peng et al., 2020), the sequencing of 16S DNA fragments that we carried out revealed significant differences in bacterial composition between inocula prepared from feces or milk depending on the donors' phenotype. Some of the differences observed concerned bacterial populations already associated in the literature with overweight or metabolic syndrome. For example, our LEfSe analysis indicated that OR inocula were typified by *Clostridiaceae 1* which were previously reported to be more abundant in the lean than overweight children (Golloso-Gubat et al., 2020) and which includes *Clostridium butyricum*, a more abundant species in OR than in OP male rats in the studies of Obanda et al. (2020). Similarly, we identified *Desulfovibrionaceae* as a hallmark of fecal inoculum from OP dams, which matched previous reports associating this family with obesity (Petersen et al., 2019) or dyslipidemia (Marungruang et al., 2018).

However, it probably is simplistic to expect similarities between studies regarding associations between bacterial populations and pathophysiological states when bacterial

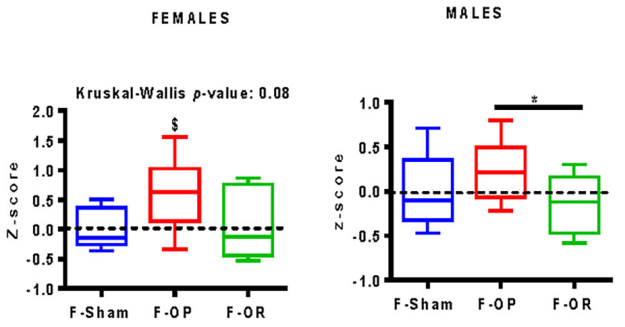

FIGURE 11 | Integrative calculated behavioral *z*-score of females (left, $n = 7–9$) and males (right, $n = 9–11$), data are median and minimum–maximum values; $^{*}p < 0.05$ indicates difference between groups, Kruskal–Wallis and *post hoc* Dunn's test, $^{\$}p < 0.05$ indicates difference from the reference score value (zero), Wilcoxon signed rank test.

composition is obtained by sequencing 16S DNA fragment: because of the risks of discordant identifications when using different databases [or different updated versions (Edgar, 2018)] and because of the inadequacy of 16S DNA fragments sequencing to provide appropriate taxonomic resolution, namely at the species or strain levels (Johnson et al., 2019). Indeed, higher taxonomic levels may include both positively correlated members and negatively correlated ones for a given physiological situation. Such a case is observed for the genus *Parabacteroides* in our study. Among the two OTUs that make it up (one identified as an unknown species, the other one associated with a multiplicity of identities), one was recognized as typical of OR fecal inoculum whereas the other one as typical of OP fecal inoculum. In this case, the genus level, and even less the family one, is obviously not appropriate to draw any conclusion, and identification of bacterial biomarkers would require a more detailed characterization of the bacterial composition of samples, e.g., after sequencing of the entire 16S DNA or after shotgun metagenome analysis.

Nevertheless, 16S DNA fragments sequencing is perfectly suited to establish the existence of differences in bacterial compositions, and those we observed between OP and OR inocula validated the animal model we had chosen to study the long-term consequences of vertical mother–neonate transfers of maternal microbiota.

Daily transfers from delivery to PND15 of these inocula did induce changes of cecocolonic microbiota in F344 recipient pups, but such impact was clearly detectable only on PND21. This observation raises two issues. First, the finding of a clear impact 6 days after the end of the inocula transfer while no difference was observed at PND11, i.e., during the exact period when transfers were applied on a daily basis, is surprising at first glance. Since the total amount of bacteria transferred remained constant throughout the transfer period (cf. **Supplementary Table 1**), it rather likely reflects the delay necessary for transferred bacteria to colonize the gut of the recipient pups, including the time required to produce a favorable environment particularly in terms of redox potential since unlike the first natural colonizers, the bacteria transferred were predominantly anaerobes from fecal inoculum (Pesti, 1979). Second, the difference in composition induced by transfers in recipient pups had vanished by the time they reached adulthood. Based on the longitudinal follow-up we performed, using feces collected at PND60 and PND130 (**Supplementary Figures 4A–D**), the microbiotal differences between transferred and control animals were minimal as early as PND60. This fading overtime was already observed in our earlier studies using neonatal prebiotic supplementations to induce early microbiological changes (i.e., prior to full weaning) (Morel et al., 2015; Le Drean et al., 2019). Our manipulations of the microbiota composition might in fact have been applied too early as they were stopped before weaning, a time when intestinal microbiota is described as unstable (Schloss et al., 2012) as a result of the change in the nature of the food consumed (see Laforest-Lapointe and Arrieta, 2017 for a review). This instability also stems from the intestinal maturation process which continues at the same time and affects both the immune and epithelial components of the colon, both of which being involved in the control of bacteria engraftment (Biol-N'garagba and Louisot, 2003; Zhang et al., 2015).

Nevertheless, although they had no long-lasting impact on the composition of the microbiota, the neonatal transfers we performed did affect recipients' physiology.

Feeding Behavior Phenotype of Fisher Recipients Varied According to OP or OR Microbiota Transfers

The multiple tests used in the present study were intended to reveal differences that would reflect traits of eating behavior predisposing transferred pups to obesity: two tests to measure reward-seeking behavior, the SD to HED switch to reveal the ability to regulate the intake of a high-energy food, the measure of the occurrence of satiety to inform about the postprandial state, and the 24 h feeding pattern to inform about night and day eating characteristics.

Significant behavioral differences were observed between groups depending on each test and according to sex. Because eating behavior is complex, we chose to use an integrated behavioral *z*-score that consolidates most relevant items of the various tests. This composite behavioral *z*-score indicates how many standard deviations (s) an observation (X) is above or below the mean of a control group (m), i.e., $Z = (X - m)/s$ as previously used to define anxiety- and depressive-like state in mice by the use of complementary tests (Guilloux et al., 2011; Mir et al., 2020). Regarding the behavioral items used to calculate this score, a value significantly above zero is in favor of a higher risk to develop an over-eating disorder predisposing to obesity and a value significantly below zero is associated with a normal-eating behavior potentially protecting against obesity. The integrated behavioral *z*-score of females F-OP showed a risk of eating behavior which includes higher food consumption and higher reward seeking behavior. For males of the F-OP group, the integrated behavioral *z*-score was significantly higher than that of F-OR counterparts, conferring to the former a riskier eating behavior.

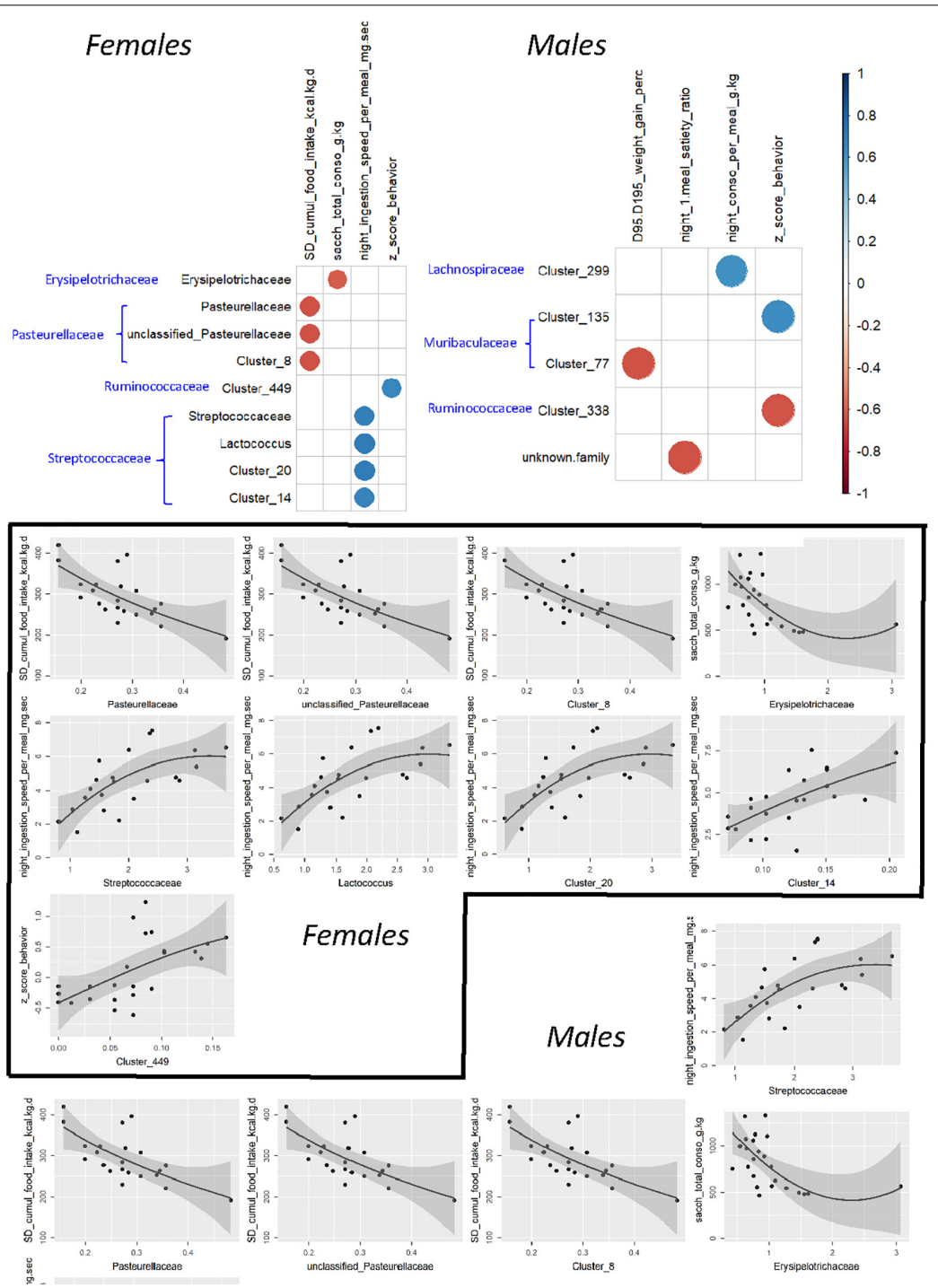

FIGURE 12 | Correlograms showing significant ($\alpha < 0.01$) Spearman rank correlations among selected components of eating behavior and the relative abundances of some bacterial taxa in F344 recipients and corresponding scatter plots, using individual data from PND200 female and male animals. In all correlograms, positive correlations are displayed in blue and negative correlations in red as scaled on the right. The black line and associated gray zone represent a LOESS smoother and the 95% confidence interval, respectively.

In the sweet and fat preference tests, all groups demonstrated a strong preference for saccharine and corn oil, and therefore the test did not discriminate between groups. However, male and female F-OR had a lower total intake of tasty liquids after 3 days of presentation. So far, little is known on the relationship between taste perception, food preferences, and microbiota, but

a few studies suggest that rodent strains created through selective pressure based on taste, the Occidental low- and high-saccharin–consuming rats, outbred on the basis of saccharin intake, harbor different microbial communities (Lyte et al., 2016). Whether the microbiota is causally related to the taste phenotype remains to be determined. The lower total intake of tasty or fatty liquid may reflect the onset of a satiety signal, not dependent on calories, which appeared faster in the F-OR rats and that may be linked with oral or intestinal microbiota composition. Interestingly in gastric bypass models of rats, a similar decrease in voluntary consumption of tasty solutions is reported (Mathes et al., 2015). This result underlines the importance of signals of intestinal origin in the regulation of the consumption of tasty foods, but the involvement of the microbiota remains to be established. In our study, the hedonic component of sugar/fat taste was not altered but postoral signals may differ between the groups to modulate potent negative consequence of an excess intake of these palatable foods (e.g., visceral pain).

In our study, the motivation test revealed a striking finding: Fisher rats that had been transferred with microbiota, regardless of the donor, performed better in the straight alley tests than F-Sham during the 10-day trials (data not shown) and during the final test, and in addition, F-OP performed better than F-OR in the final test, testifying a higher motivation to consume the food for the first group. It has been recently documented that change in gut microbe diversity and richness influence serotoninergic, GABAergic, noradrenergic, and dopaminergic neurotransmission, the latter being the key modulator of decision making, motivation, reward, and memory (Gonzalez-Arancibia et al., 2019). There is some evidence that microbiota-gut-brain axis is the key to the pathophysiology of several neuropsychiatric disorders involving dopaminergic neurotransmission, and many transient and persistent inhabitants of the gut, including *Escherichia coli* or *Enterococcus faecium* have been shown to manufacture DA (Lyte and Lyte, 2019) though availability of such DA to the brain is debated.

Satiety refers to the result of satiation that ends an eating sequence and is a transitional period separating meals. Meal size is controlled by vagal afferent nerve which integrates satiety signals from the enteroendocrine cells to the NTS in the brain stem (Berthoud, 2006). Vagal terminals end in the lamina propria so that they can sense bacterial metabolites or byproducts to modulate food intake, as illustrated by LPS-induced hyperphagia which is probably mediated by vagal TLR4 receptors. Microbiota-induced alteration of the production of satiety gastrointestinal peptides, resulting in increased food intake, has been well documented in obesity (review Gomes et al., 2018). The role of the microbiota *per se*, apart from the confounding effect of diet, on satiety (or food intake) is less well documented. In the OP-OR rodent model used in the present study, food/caloric intake has been characterized mostly on males without any association with the composition of gut microbiota. When fed HED, OP rats eat significantly more food than OR rats (Levin, 1999; Ricci and Levin, 2003; Obanda et al., 2018, 2020), but one work showed a higher food intake in OR compared with OP male rats (Levin et al., 1986). In females, only one study showed that before gestation female OP eat and weigh more than OR ones on HED (Frihauf et al., 2016). In the current work, we explored feeding behavior using multiple tests allowing fine-tune analysis of satiety, and, taken together, several items of these tests are moving in the same direction of an altered satiety in F-OP groups.

What Are the Cues for Crosstalk Between Microbiota and Key Elements of Feeding Behavior?

Our search for associations between bacterial abundances and variation in eating behavior indicators revealed different associations, mainly for abundances quantified at PND21. Although not proving a causal relationship, these associations do support the existence of an impact of certain bacterial populations on eating behavior, which would have occurred mainly in the neonatal period. Such influence could be exerted through different pathways. First, intestinal epithelium development and function could be affected during early stage (Sommer et al., 2015), such as the enteroendocrine cells, which are known to modulate food intake, mainly through gastrointestinal peptide secretion (Martin et al., 2019). Furthermore, early microbiota could influence host metabolism by modulating peripheral organ programming such as the pancreas, liver, adipose tissue, or muscles, also with long-term consequences (Backhed, 2011). In turn, these organs influence food intake *via* endocrine and/or immune and/or vagal routes (McDermott et al., 2006; Abdalla, 2017; Browning et al., 2017). However, in this work, metabolic plasmatic markers (data not shown) were not significantly different between groups, neither at weaning nor at adulthood. The present results tend to rule out the metabolic endocrine route, but a more complete characterization of the energetic regulation is required to conclude on that aspect.

More likely, an early modulation of neurodevelopment which could lead to long-term consequences on brain structure and function may be suspected. Whether early microbiota participates in the development of the central nervous system has been proposed in a series of publications and examined through the observation that key events as microglia proliferation, synaptogenesis, and pruning are impacted in germ-free mice, after antibiotics treatment or after specific fecal transplantation and result in behavioral modifications as anxiety-like symptoms, depression, or autism spectrum disorder (Goyal et al., 2015; Jena et al., 2020; Morais et al., 2020). Food intake regulation is the result of complex interplays between a large number of structures; therefore, to deepen our knowledge on the impact of early microbiota transfer, we need to go further and perform a detailed anatomical, transcriptomic, and functional analysis of the brain structures (VTA, NAc, HT, and NTS) and the different element of the gut–brain communication that can be disturbed according to the existing microbiota.

Since our work is the first to establish the proof of concept that the early intestinal microbiota can influence eating behavior later in life, the mechanistic pathways or mediators relaying the impact of the early microbiota on the central circuitries that regulate eating behavior are obviously unknown. However, they are probably close to those suspected as involved in the interaction between microbiota and neurodevelopment, which

rely mainly on the production of specific microbiotal metabolites such as the SCFA or neuromediators and the microbiota-induced mediation of immune signaling (see Warner, 2019 for review).

With this respect, the association we observed in males between the abundance of UBA1819 (a taxon affiliated to the *Faecalibacterium* sp.) which was significantly more abundant in the F-OR group at weaning and the total fat consumption during taste preference tests is of particular interest given the immunomodulatory potential of some species belonging to this genus (Delgado et al., 2020). Similarly, the correlation that we observe for females between the abundance of cluster 10 (affiliated to *Enterobacteriaceae*) at PND21 and *z*-score is intriguing since *E. coli* synthetize dopamine (see Lyte and Lyte, 2019 for review) while this property is present in less than 5% of the bacterial genomes representative of the human intestinal microbiota (Valles-Colomer et al., 2019). Further investigation of these tracks requires more precise bacterial identification, if possible at the strain level, since differences in physiological effects have been observed at this taxonomic scale, particularly in the case of appetite regulation by the intestinal microbiota (Chagwedera et al., 2019). These precisions can be obtained by applying a metagenomic study, which will also make it possible to search for associations with particular functions. Interestingly in this respect, Chagwedera et al. (2019) showed that to be effective, their sole effective strain had to be administered to mice with other microbiotal components but not in pure culture. This feature is most likely related to the fact that a gene coding for a bacteriocin has been identified among the specific genes of this strain. This reminds that correlations do not necessarily reveal direct relations between bacteria and mechanisms supposedly involved in host physiology regulation.

CONCLUSION

The current study clearly supports the hypothesis that the transfer of microbiota from mother to pup in early neonatal life can impact eating behavior at adulthood. Our study is the first to go beyond food intake and body weight gain to examine in the same animals and across time the various aspect of eating behavior. Functional metagenomics and offspring metabolomics should help explain how microbiotal activity can alter physiology with regard to eating behavior regulation. Whether mother-to-child microbiotal transfer plays a role in the transmission of metabolic risk clearly deserves further investigation in humans.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The 16S rRNA gene sequences analyzed in this study are available through the National Center for Biotechnology Information (NCBI) Sequence Read Archive Dataset under accession number SRP310757/PRJNA714462, accessible with the following link: https://www.ncbi.nlm.nih.gov/sra/PRJNA714462.

ETHICS STATEMENT

The animal study was reviewed and approved by Protocols were approved by the local Committee on the Ethics in Animal Experiments of Pays de la Loire (France) and the French Ministry of Research (APAFIS#6617-2016072916395797-v6). Animal facility is registered by the French Veterinary Department as A44276.

AUTHOR CONTRIBUTIONS

PP, CM, GL, CH, and EL designed the research. CM, GL, PP, A-LP, HB, TM, AP, and EL performed the research. PP, CM, GL, and EL supervised the research. CM, GL, PP, A-LP, HB, TM, and EL analyzed the data. PP, CM, GL, and A-LP wrote the manuscript. All authors edited and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.672224/full#supplementary-material

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Tau expression and phosphorylation in enteroendocrine cells

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Background and objective: There is mounting evidence to suggest that the gut-brain axis is involved in the development of Parkinson's disease (PD). In this regard, the enteroendocrine cells (EEC), which faces the gut lumen and are connected with both enteric neurons and glial cells have received growing attention. The recent observation showing that these cells express alpha-synuclein, a presynaptic neuronal protein genetically and neuropathologically linked to PD came to reinforce the assumption that EEC might be a key component of the neural circuit between the gut lumen and the brain for the bottom-up propagation of PD pathology. Besides alpha-synuclein, tau is another key protein involved in neurodegeneration and converging evidences indicate that there is an interplay between these two proteins at both molecular and pathological levels. There are no existing studies on tau in EEC and therefore we set out to examine the isoform profile and phosphorylation state of tau in these cells.

Methods: Surgical specimens of human colon from control subjects were analyzed by immunohistochemistry using a panel of anti-tau antibodies together with chromogranin A and Glucagon-like peptide-1 (two EEC markers) antibodies. To investigate tau expression further, two EEC lines, namely GLUTag and NCI-H716 were analyzed by Western blot with pan-tau and tau isoform specific antibodies and by RT-PCR. Lambda phosphatase treatment was used to study tau phosphorylation in both cell lines. Eventually, GLUTag were treated with propionate and butyrate, two short chain fatty acids known to sense EEC, and analyzed at different time points by Western blot with an antibody specific for tau phosphorylated at Thr205.

Results: We found that tau is expressed and phosphorylated in EEC in adult human colon and that both EEC lines mainly express two tau isoforms that are phosphorylated under basal condition. Both propionate and butyrate regulated tau phosphorylation state by decreasing its phosphorylation at Thr205.

Conclusion and inference: Our study is the first to characterize tau in human EEC and in EEC lines. As a whole, our findings provide a basis to unravel the functions of tau in EEC and to further investigate the possibility of pathological changes in tauopathies and synucleinopathies.

KEYWORDS

enteroendocrine cells, tau, short-chain fatty acids, gut-brain axis, tauopathies, gastrointestinal tract

Introduction

The enteroendocrine cells (EEC), which are scatterly distributed along the entire gastrointestinal (GI) mucosa representing around 1% of the total gut epithelium cell population, are key components of the gut-brain axis. They are classically regarded as specialized hormone-secreting cells with an apical surface that is exposed to gut lumen and a basal portion that contains secretory granules (Gribble and Reimann, 2019). Such an orientation allows EEC to respond to intraluminal signals such as nutrients or microbiota-derived metabolites. This aspect has been particularly well documented for short-chain fatty acids (SCFA), such as butyrate and propionate, which induce the release of the peptide hormone YY (PYY) (Larraufie et al., 2018). Morphologically, EEC were shown to exhibit neuron-like features including the expression of pre- and post-synaptic proteins together with the presence of neurite-like processes, called neuropods (Bohórquez et al., 2015). These neuropods are in close contact with the two components of the enteric nervous system (ENS), namely enteric glial cells (Bohórquez et al., 2014) and enteric neurons (Chandra et al., 2017) as well as vagal neurons (Kaelberer et al., 2018). The recent findings, which showed that EEC contain alpha-synuclein (Chandra et al., 2017), a presynaptic neuronal protein genetically and neuropathologically linked to Parkinson's disease (PD) led to the assumption that they might be involved in the development of PD. By facing the gut lumen and being directly connected with alpha-synuclein positive enteric neurons (Chandra et al., 2017), the EEC might be a key component of the neural circuit between the gut lumen and the brain for the bottom-up propagation of PD pathology as initially hypothesized by Braak et al. (2006) and more recently by Borghammer and Van Den Berge (2019).

Alpha-synuclein accumulates in a group of neurodegenerative diseases collectively known as synucleinopathies with PD being the most common, while the accumulation of tau is a defining feature of tauopathies classically found in the brains of patients with Alzheimer's disease (AD) or progressive supranuclear palsy (PSP) (Dugger and Dickson, 2017). Nevertheless, in numerous cases alpha-synuclein positive inclusions are also described in tauopathies and *vice versa*, suggesting a co-existence or crosstalk of these proteinopathies (Arai et al., 2001; Coughlin et al., 2019). Tau, like alpha-synuclein is expressed by enteric neurons (Lionnet et al., 2018), thereby suggesting that enteric tau might be involved in neurodegenerative disorders and/or enteric neuropathies (Derkinderen et al., 2021). In contrast to alpha-synuclein, no data are available about the distribution and phosphorylation pattern of tau isoforms in EEC. Here, we first examined the expression levels of tau isoforms and their phosphorylation profile in full thickness segments of human colon and in EEC lines. We then studied the regulation of tau phosphorylation by SCFA in EEC. Our results show the presence of phosphorylated tau in both mouse and human colonic EEC and the expression of one main tau isoform in EEC lines. We also show that EEC tau is phosphorylated under basal condition and that its phosphorylation state can be modified by SCFA. These data provide the first detailed characterization of EEC tau in human adult colonic tissues and in cell lines. Further investigation of tau modifications in EEC in pathological conditions may provide valuable information about the possible role of EEC tau in neurodegenerative diseases.

Materials and methods

Human, mouse, and rat tissues

Specimens of human colon were obtained from eight neurologically unimpaired subjects who underwent colon resection for colorectal cancer (5 men, 71 ± 7.6 years). For all tissues specimens, sampling was performed in macroscopically normal segments of uninvolved resection margins. The sampling of human colon was approved by the Fédération des Biothèques of the University Hospital of Nantes, according to the guidelines of the French Ethics Committee for Research on Humans and registered under the no. DC-2008-402. Written informed consent was obtained from each subject. Hippocampi were taken from a 2-month-old wild type C57BL/6J mouse and sciatic nerve sections were taken from one pregnant Sprague-Dawley rats (used for the generation of primary culture of rat ENS in our lab) in order to identify tau isoforms in GLUTag cells and to serve as a positive control for big tau experiments, respectively. These samples were stored at −80°C until further analysis by Western blot.

EEC lines and reagents

NCI-H716 cells (ATCC, LGC Standards, Molsheim, France, CCL-251) were maintained in RPMI-1640 (Gibco, Life Technologies, Villebon-sur-Yvette, France) whereas GLUTag (a gift from Professor Daniel J. Drucker of University of Toronto) and Caco-2 cells were maintained in DMEM (Gibco), all supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin (all from Merck-Sigma, Saint-Quentin Fallavier, France) in a humidified incubator at 37 °C with 5% of CO_2. Propionate and butyrate were from Merck-Sigma.

Dephosphorylation of tissues and cell lysates

For dephosphorylation experiments, cells and hippocampi were homogenized in a buffer containing 100 mM NaCl and 50 mM Tris-Cl at pH 7.4 with 1% (v/v) IGEPAL® CA-630 (ThermoFisher, Saint-Herblain, France) and a protease inhibitors cocktail without EDTA (Roche, Neuilly sur Seine, France) using either a "Precellys 24" (Bertin technologies, St Quentin-en-Yvelines, France) tissue homogenizer and followed by sonication with "vibracell 75 186" device (Sonics, Newton, CT, USA). Homogenates were centrifuged at 16,100 *g* for 20 min at 4°C with an Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany), sonicated for 10 s and protein amounts normalized following a bicinchoninic acid protein assay (ThermoFisher). Samples were diluted to 1.0 mg/ml protein using homogenization buffer and incubated with 20 U/μl lambda phosphatase in $MnCl_2$ and enzyme buffer as supplied with the lambda protein phosphatase kit (New England Biolabs, Evry-Courcouronnes, France) for 3 h at 30°C. The reaction was stopped by the addition of sample buffer (Life Technologies) and heating to 95°C for 5 min. Control samples were treated identically without the addition of lambda phosphatase.

SDS-PAGE and Western blot

For dephosphorylation experiments, cells or tissues were processed as described above. For experiments that did not require dephosphorylation, cells or tissues were lysed in RIPA lysis buffer (Merck Millipore, Fontenay sous Bois, France) containing 2 mM orthovanadate (Merck-Sigma, Molsheim, France), phosphatase inhibitor cocktail II (Merck-Sigma) and a protease inhibitors cocktail (Merck-Sigma). Western blots were performed as we previously described using NuPAGE™ 10% Bis-Tris Protein Gels (Life Technologies). The primary anti-tau antibodies used are listed in **Table 1**. β-Actin antibodies (Abcam, France, 1:1,000 dilution) were used as loading control. Tau ladder (six human tau recombinant isoforms, Sigma-Merck) was used to identify tau isoforms in NCI-H716 cells. For quantification, the relevant immunoreactive bands were quantified with laser-scanning densitometry and analyzed with Image Lab software (Biorad, Marnes-la-Coquette, France) or ImageJ software (NIH; version 1.51). To allow comparison between different films, the density of the bands was expressed as a percentage of the average of controls. pThr205 tau immunoreactive bands were measured, normalized to the optical densities of total tau, and expressed as percentage of controls.

Immunofluorescence

Human colonic tissues were embedded in paraffin using an embedding station (LEICA EG1150C) and sections (3 μm) were cut using a microtome (LEICA RM2255). The sections were deparaffinized by bathing twice in xylene (for 5 min each) and taken through graded concentrations of ethanol (100, 95, 70, and 50%, respectively, for 3 min each). After a rinse in distilled water, slides were washed in PBS and antigen retrieval was performed using a Sodium Citrate solution (2.94 g Sodium Citrate Tribase; 1 L distilled water; 500 μl Tween 20; pH 6) at 95°C for 20 min. Slides were incubated in NH_4Cl (100 mM) for 15 min before incubation in PBS-0.5% triton X-100 (Merck-Sigma) for 1 h and blocking for 2 h in 10% (v/v) horse serum in PBS-0.5% triton X-100. Primary antibodies (**Table 1**) were incubated overnight at 4°C, and following washing, secondary antibodies were added for 2 h at room temperature. Secondary antibodies were anti-mouse Biotin (A24522, Thermo Fisher Scientific), Alexa Fluor 647-conjugated goat anti-rabbit (711-606-152, Jackson Immunoresearch, Ely, UK), Alexa Fluor 568-conjugated Streptavidin (S11226, Invitrogen, Thermo Fisher Scientific), Alexa Fluor 568-conjugated goat anti-mouse (Molecular Probes, Thermo Scientific). DAPI (1:10,000) was added to counterstain nuclei. Tissues sections were mounted in Prolong Gold anti-fading medium (Molecular Probes, Thermo Scientific). Images were acquired with a Zeiss, Axio Imager M2m fluorescence microscope coupled to a digital camera (Axiocam 503 mono).

RNA extraction and RT-PCR

Total RNA extraction from EEC and Caco-2 cells was carried out using a QIAamp RNA Blood Mini kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. 1 μg RNA per sample was reverse transcribed using a Superscript III reverse transcriptase assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. To examine the alternate splicing of the microtubule binding domain repeat region encoded by exon 10, primers were used that specifically recognize mouse or human exons 9 and 11 as described by Duff et al. (2000). Primer sequences were: mouse exon 9F 5′-CCCCCTAAGTCACCATCAGCTAGT, mouse exon 11R 5′-CACTTTGCTCAGGTCCACCGGC, human exon 9F 5′-CTCCAAAATCAGGGGATCGC, human exon 11R 5′-CCTTGCTCAGGTCAACTGGT. Splicing around the N terminal insert domain encoded by exons 2 and 3 was detected using primers that recognize exons 1 and 5. Primer sequences used were: mouse exon 1F 5′-TCCGCTGTCCTCTTCTGTC, mouse exon 5R 5′-TTCTCGTCATTTCCTGTCC, human exon 1F 5′-TGAACCAGGATGGCTGAGC, human exon 5R 5′-TTGTCATCGCTTCCAGTCC. Annealing temperatures were 64°C (all MAPT primers), 62°C (M1F/M5R) and 68°C (M9F/M11R). Thirty-five reaction cycles were used for all. Mouse and human-specific RT-PCR products were analyzed by agarose gel electrophoresis. Products corresponding to exon 10+ tau mRNA (4R) are 390 base pairs (bp), while products corresponding to exon 10- mRNA (3R) are 297 bp. RT-PCR products containing tau mRNA with exons 2 and 3 (2N) are 428 bp, 2 + 3- mRNA products (1N) are 341 bp, and 2–3- mRNA products (0N) are 253 bp.

Statistics

All quantitative data shown are mean ± standard error of the mean (SEM). Mann–Whitney test or Kruskal–Wallis then Dunn's multiple comparisons tests were performed for two or multiple groups comparison, respectively. Differences were deemed statistically significant if $p < 0.05$. GraphPad Prism software version

9.1.1 (GraphPad Software Inc., La Jolla, CA, United States) was used for statistical analyses and for designing figures.

Results

Human colonic EEC express tau

To determine if tau is expressed in EEC, we examined chromogranin A and glucagon-like peptide 1 (GLP-1)-containing cells of the human colon. Chromogranin A is an acidic glycoprotein located in secretory vesicles of endocrine cells, which is classically used as a pan-EEC marker (Wilson and Lloyd, 1984), whereas GLP-1 is a marker of L-type EEC (Eissele et al., 1992), the most abundant EEC population in the human colon (Latorre et al., 2016). Using the pan-tau antibody A0024, we identified tau in GLP-1-positive cells in the epithelial lining (**Figure 1A**). When human colonic tissue was immunolabelled with isoform-specific tau antibodies, we showed that 3R-tau was expressed in chromogranin A immunoreactive cells (**Figure 1B**); no specific staining was observed when a 4R-tau was used (data not shown). Tau phosphorylation at multiple serine and threonine sites is the predominant mechanism by which its biological activity is regulated (Guo et al., 2017). We therefore examined the phosphorylation state of tau with a phospho-specific antibody that detect tau phosphorylated at Ser396 and showed that phospho-tau was observed within chromogranin A-positive cells in colon epithelium (**Figure 1C**). Approximately 73 and 80% of chromogranin A immunoreactive cells in the colon contained 3R- and phosphorylated-tau, respectively (**Table 2**). When taken together, these results show that tau is expressed and phosphorylated in EEC in human colon, with 3R-tau likely being most abundant isoforms.

Tau is expressed and phosphorylated in EEC lines

There are multiple cell lines used as models for EEC research, including GLUTag, a mouse endocrine tumor-adherent cell line and NCI-H716, a human-derived suspension cell line. These two cell lines have been widely used as models for GLP-1–producing L-cells and proved to be useful for *in vitro* screening bioassays (Goldspink et al., 2018). As a first approach, we compared the banding pattern on Western blots of total tau as evaluated with the A0024 pan-tau antibody between non-dephosphorylated lysates of GLUTag cells and hippocampus of 2-month-old mouse. Total tau antibody detected several bands in 2-month-old mouse brain migrating between 50 and 60 kDa (**Figure 2A**). In GLUTag cells, the observed banding pattern was markedly different with a doublet of 48 and 50 kDa bands, the latter showing the most intense labeling (**Figure 2A**). In adult mouse brain, primary CNS/ENS neurons and neuronal cell lines, tau isoforms are phosphorylated on multiple tau and serine residues resulting in reduced electrophoretic mobility on SDS-PAGE compared to non-phosphorylated tau (Goedert et al., 1992a; Davis et al., 1995; Lionnet et al., 2018). In order to determine the phosphorylation state of tau in GLUTag cells, cell lysates treated or not with lambda phosphatase (Davis et al., 1995) were analyzed by Western blot using three different pan-tau antibodies. Treatment with lambda phosphatase caused tau dephosphorylation, as evidenced by a significant downward shift in mobility of the tau doublet detected with either the pan-Tau A0024, D1M9X, or Tau-1 (**Figure 2B**). Of note, this downward shift was associated with increased Tau immunoreactivity when the Tau-1 antibody was used (**Figure 2B**). These findings are in line with previous observations showing that Tau-1 binds preferentially to tau when dephosphorylated at serine residues 195, 198, 199, and 202 (Liu et al., 1993; Szendrei et al., 1993). In order to determine which tau isoforms are expressed in GLUTag cells, we used two commercially available isoform-specific tau antibodies directed against 3R and 4R-tau, which have been shown to be highly specific in a recent comprehensive study that tested the specificity of tau antibodies using immunoblotting (Ercan et al., 2017). GLUTag lysates were compared to dephosphorylated brain samples (hippocampus of 2-month-old wild-type mice), which express all 4R isoforms and to a lesser extent the 0N3R isoform (McMillan et al., 2008; Liu and Götz, 2013; **Figure 2C**). After dephosphorylation, the 3R and 4R antibodies detected one single band in cell lysates that comigrated with 0N3R and 0N4R in the hippocampus, respectively (McMillan et al., 2008; Liu and Götz, 2013; **Figure 2C**). When taken together, these results show that 0N3R and 0N4R are the two main tau isoforms that are expressed in GLUTag cells and these two isoforms are phosphorylated under basal conditions.

TABLE 1 The name, specificity, epitope, source, and dilution of the antibodies used in this study are shown.

Name	Specificity	Epitope (a.a.)	Source and dilution
A0024 Tau	All tau isoforms	243–441 (2N4R)	Dako, rp (WB 1:1,000; IF 1:500)
Tau-1	All tau isoforms	189–207 (2N4R)	Merck, mm, clone PC1C6 (WB 1:2,000; IF 1:500 biotin)
D1M9X	All tau isoforms	Around D430 (2N4R)	Cell Signaling, rm #46687 (WB 1:1,000)
Anti tau RD3	3R tau Isoforms	267–282 (2N3R)	Merck, mm, clone 8E6 (WB 1:1,000; IF 1:500)
Anti tau RD4	4R tau isoforms	275–291 (2N4R)	Merck, mm, clone 1E1/A6 (WB 1:1,000)
Anti 4R-tau	4R tau isoforms	Around 273 (2N4R)	Cell Signaling, rm #30328 (WB 1:2,000; IF 1:200)
PHF13	Tau Ⓟ S396	Tau Ⓟ S396	Cell Signaling, mm (WB 1:1,000; IF 1:200, biotin)
pThr205	Tau Ⓟ T205	Tau Ⓟ T205	Abcam, rm, #EPR2403(2) (WB 1:1,000)
ChrA	SP-1 chromogranin A		Spring Bioscience, rp, #E1520 (IF: 1:1,000)
Glp-1	C-term Glp-1		Santa Cruz, gp, #7782 (IF 1:200)

a.a., amino-acids; IF, immunofluorescence; GP, goat polyclonal; MM, mouse monoclonal; RM, rabbit monoclonal; RP, rabbit polyclonal; WB, Western blot.

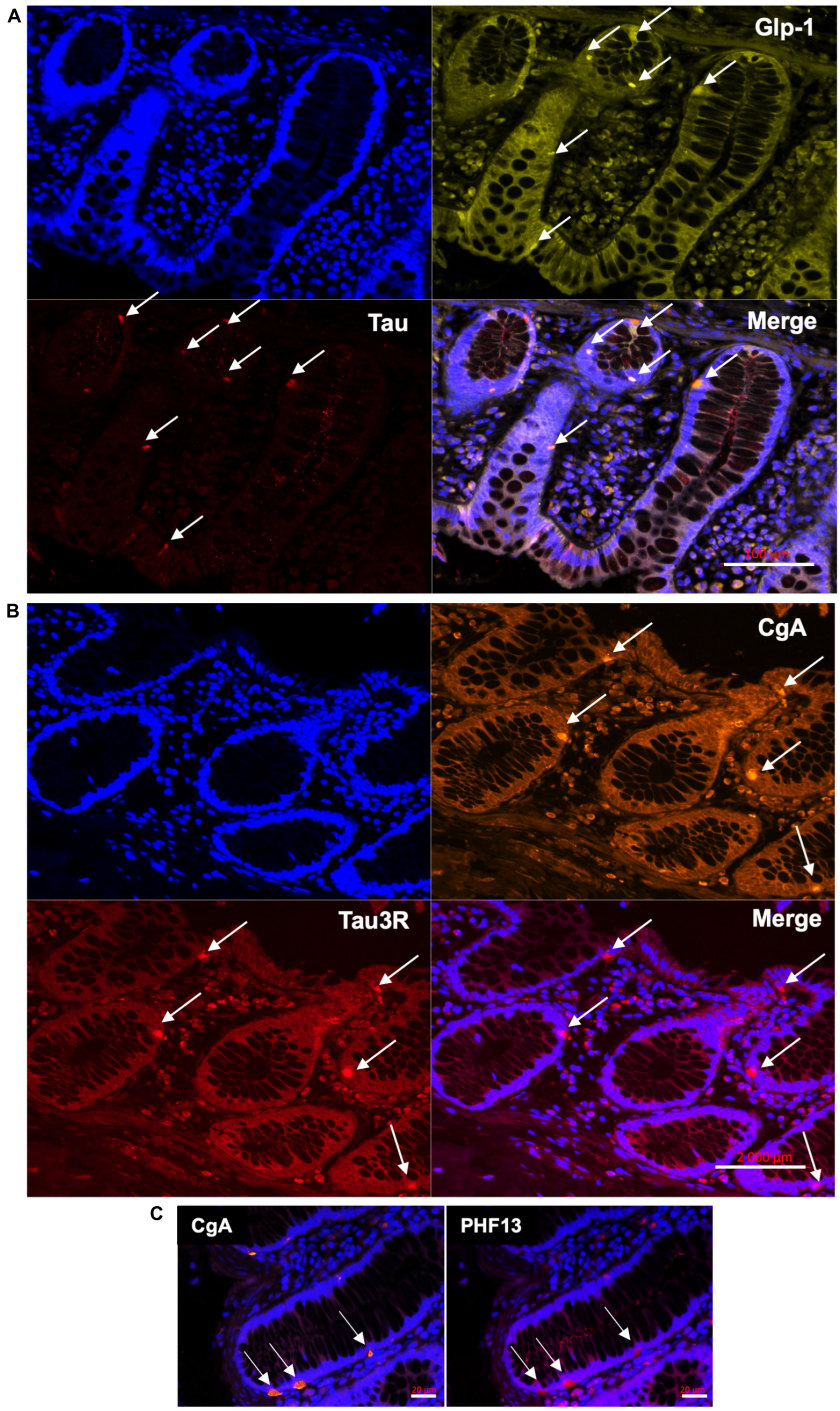

FIGURE 1
Distribution of tau in the epithelial lining in adult human colon. **(A)** Total tau antibody A0024 (Table 1) was used to detect tau in epithelium lining of human colonic samples (arrows). An antibody specific to Glucagon-like peptide 1 (GLP-1) was used to specifically label L-type EEC (arrows). DAPI was used to counterstain nuclei. Scale bar is 100 μm. **(B)** Isoform-specific antibody (tau RD3, Table 1) was used to detect 3R-tau and an antibody specific to chromogranin A (CgA) was used to specifically label EEC (arrows). DAPI was used to counterstain nuclei. Scale bar is 2 mm. **(C)** Human colonic samples were co-labeled with an antibody specific for tau phosphorylated at Ser396 (PHF13, Table 1) and an antibody specific to chromogranin A (CgA). Arrows show cells immunoreactive for both PS396 and CgA. DAPI was used to counterstain nuclei. Scale bar is 20 μm. Representative photomicrographs are shown.

Similar experiments were conducted with the NCI-H716 cell line. Treatment with lambda phosphatase caused tau dephosphorylation, as evidenced by a significant downward shift in mobility of the tau doublet detected when the pan-tau A0024 antibody was used (Figure 2D). After dephosphorylation, this tau doublet at 50 and 55 kDa comigrated with 0N3R and 1N3R/0N4R from the tau human ladder (Figure 2D). The isoform specific 3R antibody detected one major at band at 58 kDa and a fainter one

around 55 kDa in dephosphorylated samples that comigrated with 1N3R and 0N3R isoforms of recombinant human tau, respectively (**Figure 2D**). No major bands were observed when the 4R-tau antibody was used (data not shown). As a whole, these results show that 1N3R and to a lesser extent 0N3R are the two main isoforms expressed in NCI-H716 cell.

"Big" or peripheral tau is a tau isoform specifically expressed in the peripheral nervous system, including sciatic nerve (Taleghany and Oblinger, 1992). It differs from the 2N4R tau isoform by a 254 amino-acid insert located in the amino-terminal half and migrates at 110 kDa on SDS/PAGE (Goedert et al., 1992b). As shown in **Supplementary Figure 1**, the uncropped images of the Western blots performed from **Figure 2** with total tau antibodies (A0024, D1M9X, and Tau-1) did not show any additional bands at 110 kDa in either GLUTag and NCI-H716 cells, thereby suggesting that EEC do not express big tau. Additional experiments were performed with Tau-1 antibody, which has been previously shown to detect big tau (Davis and Johnson, 1999) and rat sciatic nerve to serve as positive controls (Taleghany and Oblinger, 1992). Tau-1 detected the expected low molecular weight tau isoforms around 50 kDa in GLUTag cells, however, a 110 kDa migrating band was only observed with rat sciatic nerve lysates (**Figure 2E**).

To further refine the analysis of tau isoforms in EEC, tau expression was analyzed at the mRNA level. To this end, RNA from both GLUTag and NCI-H716 was reverse transcribed to cDNA and amplified with PCR. The human epithelial cell line Caco-2 was used for comparison. Primers were designed, based on those previously described by Duff et al. (2000), to detect splicing of human and mouse tau exons 2, 3, and 10. This allowed amplification of products corresponding to 0N tau in GLUTag, 1N (and to a lesser extent 2N) in NCI-H716 (**Figures 3A, B**). No transcripts were observed in Caco-2 cells. Transcripts of 3R tau were also observed in GLUTag and NCI-H716, but not in Caco-2 cells when inclusion of exon 10 was assessed using primers specific to human tau exons 9 and 11 (**Figure 3**). Thus, the results obtained at the mRNA level confirm those obtained at the protein level and show that 0N3R and 1N3R are the mains isoforms expressed by GLUTag and NCI-H716 cells, respectively. They also show that human intestinal epithelial cells do not express tau.

Tau phosphorylation is regulated by SCFA in EEC lines

Short-chain fatty acids act as ligands for G protein coupled receptors, which are expressed in EEC, where they mediate hormone release, such as PYY and GLP-1 (reviewed in Martin-Gallausiaux et al., 2021). The phosphorylation of tau at multiple serine and threonine sites has been described in both developing and adult brain and is the predominant mechanism by which tau functions are regulated (Guo et al., 2017). This logically led us to study the effects of SCFA on tau phosphorylation in EEC. To this end, GLUTag cell lines were treated with 1 mM of either propionate or butyrate at different time points from 10 to 180 min and cell lysates were analyzed by Western blot with an antibody specific for tau phosphorylated at Thr205. Treatment with propionate or butyrate for 180 min caused tau dephosphorylation at Thr205 when compared to control conditions (**Figures 4A, B**), without significant changes in tau expression (**Figures 4A, B**).

Discussion

Tau is a microtubule-associated protein for which the physiological functions are still a topic of intense investigation (Guo et al., 2017). Additionally, in pathological conditions tau is a key player in the pathogenesis of several diseases collectively referred to as tauopathies including AD and PSP (Guo et al., 2017). In the adult brain, tau is classically described as a neuronal protein specifically localized and highly enriched in axons but precise localization studies showed that tau distribution in the mature CNS is more widespread than initially thought, with expression in the somatodendritic compartment of neurons as well as in glial cells (Kanaan and Grabinski, 2021). Besides the CNS, the presence of tau has been demonstrated in several non-neuronal cells, such as monocytes (Kim et al., 1991), lymphocytes (Kvetnoy et al., 2000), testicular spermatids (Ashman et al., 1992), podocytes (Vallés-Saiz et al., 2022), pancreatic beta cells (Maj et al., 2016) as well as in peripheral neurons, including enteric neurons (Lionnet et al., 2018). Here, we show for the first time that tau is expressed in EEC, not only in the adult human colon but also in two EEC lines, namely GLUTag and NCI-H716.

Earlier studies showed that EEC exhibit neuronal features with the presence of axon-like basal processes and the expression of neuronal proteins such as synapsin 1, PGP9.5 and neurofilaments (Bohórquez et al., 2014, 2015). Our identification of tau in EEC further expands the neuronal repertoire of EEC and echoes recent publications which showed that alpha-synuclein is also expressed in EEC (Chandra et al., 2017; Casini et al., 2021; Amorim Neto et al., 2022). The observation that alpha-synuclein EEC lie in close proximity to alpha-synuclein–expressing enteric neurons led Liddle and collaborators to posit that the EEC might be critically involved in the circuit between the gut lumen and the brain for the bottom-up propagation of PD pathology (Chandra et al., 2017). Our current findings together with our previous data showing that enteric neurons express tau (Lionnet et al., 2018) suggest that such a scenario could also occur in tauopathies. It could thus be suggested that an hitherto unidentified substance from the gut lumen could induce changes in tau conformation in EEC, leading to a prion-like spreading of tau from EEC to enteric neurons, as

TABLE 2 Estimation of tau- and phospho-tau-expressing EEC.

	Samples (*n*)	Crypts (*n*)	CgA+ (*n*)	CgA+/crypt	Tau+/CgA+ (%)
Tau3R	5	191	238	1.24 ± 0.52	73 ± 18
PHF13	6	617	507	0.82 ± 0.47	80 ± 13

The number (*n*) of tissue samples, colonic crypts, and chromogranin A immunoreactive cells (CgA+) that were analyzed are shown. The last column shows the percentage of cells immunoreactive for Tau3R and PHF13 relative to chromogranin A immunoreactive cells.

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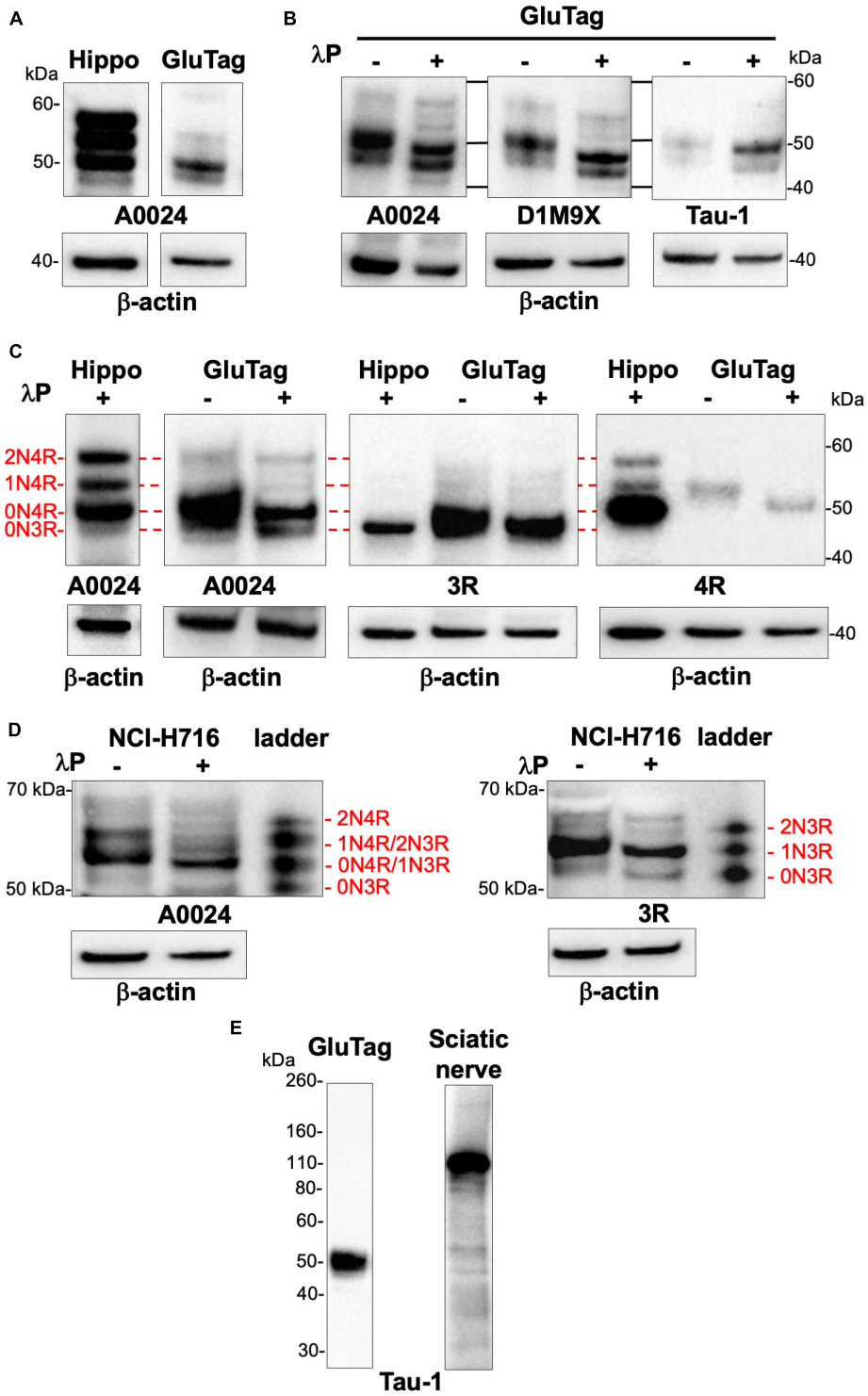

FIGURE 2
Tau protein isoforms and phosphorylation in EEC lines. **(A)** Hippocampus (2-month-old mice, Hippo) and GLUTag cell lysates were subjected to immunoblot analysis using the pan-Tau antibody A0024. **(B)** Lysates of GLUTag cell were treated (+) or not (−) with lambda phosphatase (λP) before immunoblotting with the pan-tau antibodies A0024, D1M9X, and Tau-1. **(C)** GLUTag cells lysates were treated (+) or not (−) with lambda phosphatase (λP) before immunoblotting with the tau isoform-specific antibodies 3R and 4R and the pan-tau antibody A0024. Hippocampus lysate from 2-month old mice dephosphorylated with lambda phosphatase (λP+), which contains all 4R isoforms and 0N3R isoform (McMillan et al., 2008; Liu and Götz, 2013) was used as a ladder to determine mouse tau isoform profile in GLUTag cells; the red lines show comigration. **(D)** NCI-H716 cell lysates were treated (+) or not (−) with lambda phosphatase (λP) before immunoblotting with the tau isoform-specific antibody 3R and the pan-tau antibody A0024. Tau ladder, which contains all 6 dephosphorylated human tau isoforms was used to determine tau isoform profile. **(E)** GLUTag cells lysates were subjected to immunoblot analysis using Tau-1 antibody. Rat sciatic nerve lysates were used as positive control to detect big tau. In all experiments, β-actin immunoblot was used as a loading control. The results shown in panels **(A,C)** are representative of 3 and 2 independent experiments, respectively. The results shown in panel **(B)** are representative of 7, 2, and 3 independent experiments for A0024, DM19X, and Tau-1 antibodies, respectively. The results shown in panels **(D,E)** are representative of 3 and 2 independent experiments, respectively.

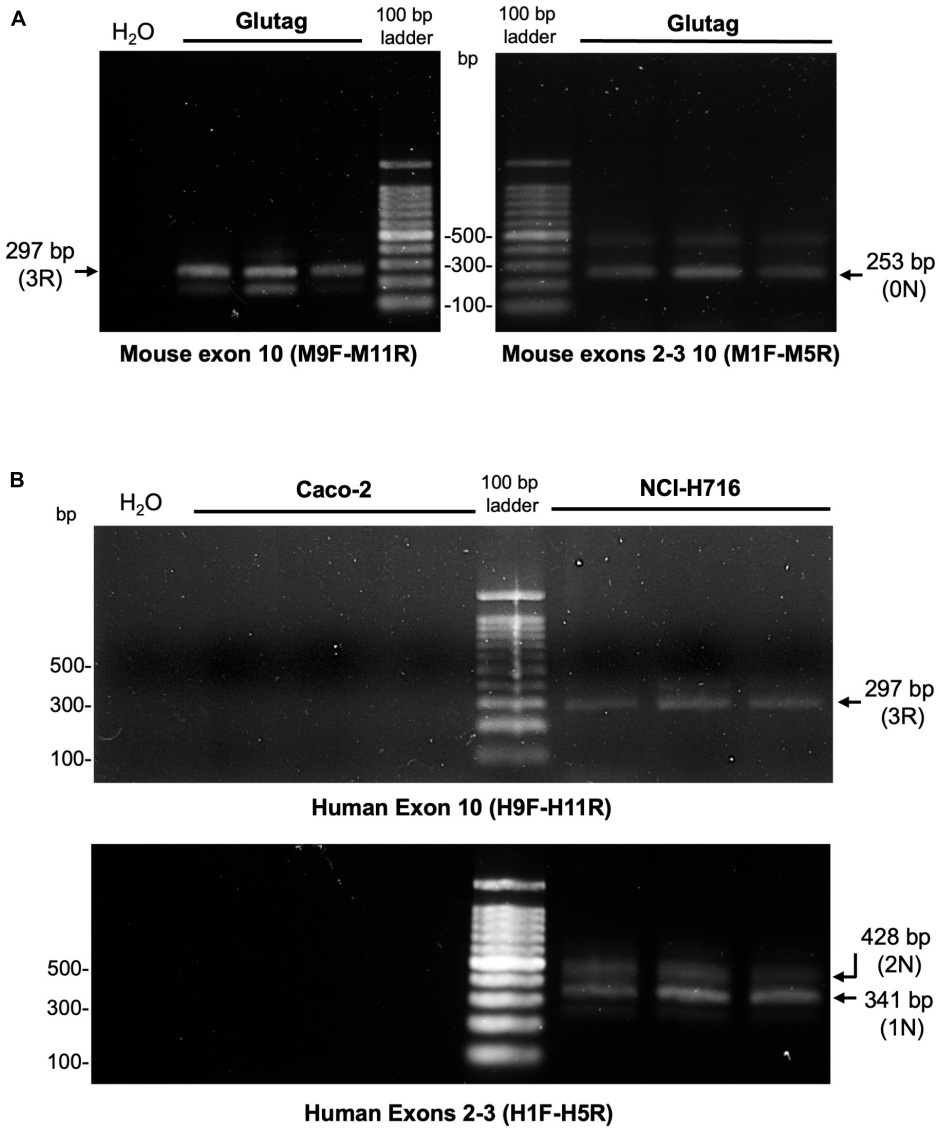

FIGURE 3
Detection of tau mRNA in EEC lines. Mtau and htau cDNA were amplified using PCR with mouse- and human-tau specific primers to detect the expression of exon 2–3- (0 N), 2 + 3- (1 N), 2 + 3 + (2 N), 10- (3R), and 10 + (4R) tau isoforms in GLUTag **(A)** and NCI-H716 cells **(B)**, respectively. Gel (2% agarose) images are shown. cDNA from colonic Caco-2 cell line were used as negative control. Numbers correspond to base pairs of a DNA ladder. The expected positions of PCR products are indicated by an arrow. *n* = 3.

already described between CNS neurons (Clavaguera et al., 2009; Frost et al., 2009). It should be however borne in mind that, unlike PD and synucleinopathies (De Guilhem De Lataillade et al., 2020), all existing studies suggest that pathological tau species are not observed in the gut of subjects with either AD or PSP (Shankle et al., 1993; Lionnet et al., 2018; Dugger et al., 2019). Further studies are therefore necessary to determine if pathological tau species are present in the gut of patients with tauopathies, either in enteric nerves or EEC (Derkinderen et al., 2021). Regarding the EEC, the identification of these potential pathological tau species in the gut could greatly benefit from novel approaches, for example by combining laser capture microdissection of EEC (Blatt and Srinivasan, 2008) with ultrasensitive amplification techniques of aggregated proteins such as real-time quaking-induced conversion (Wu et al., 2022).

Six isoforms of tau are expressed in adult human brain by alternative splicing from a single gene. Regulated inclusion of exons 2 and 3 yields tau isoforms with 0, 1, or 2 N-terminal inserts (0N, 1N, and 2N, respectively), whereas exclusion or inclusion of exon 10 leads to expression of tau isoforms with three (3R) or four (4R) microtubule-binding repeats. The various splice combinations of tau are thus abbreviated-0N3R, 0N4R, 1N3R, 1N4R, 2N3R, 2N4R-encoding six proteins isoforms ranging from 352 to 441 amino acids in length (Goedert and Jakes, 1990). In the current study, we identified 0N3R/0N4R and 0N3R/1N3R as the two main tau isoforms expressed in GLUTag and NCI-H716 cell lines. The reasons why EEC lines only express a subset of tau isoforms remains to be determined but the observation showing that the intracellular sorting of tau in different cell compartments is isoform-dependent may provide a clue (Liu and Götz, 2013). It has been indeed

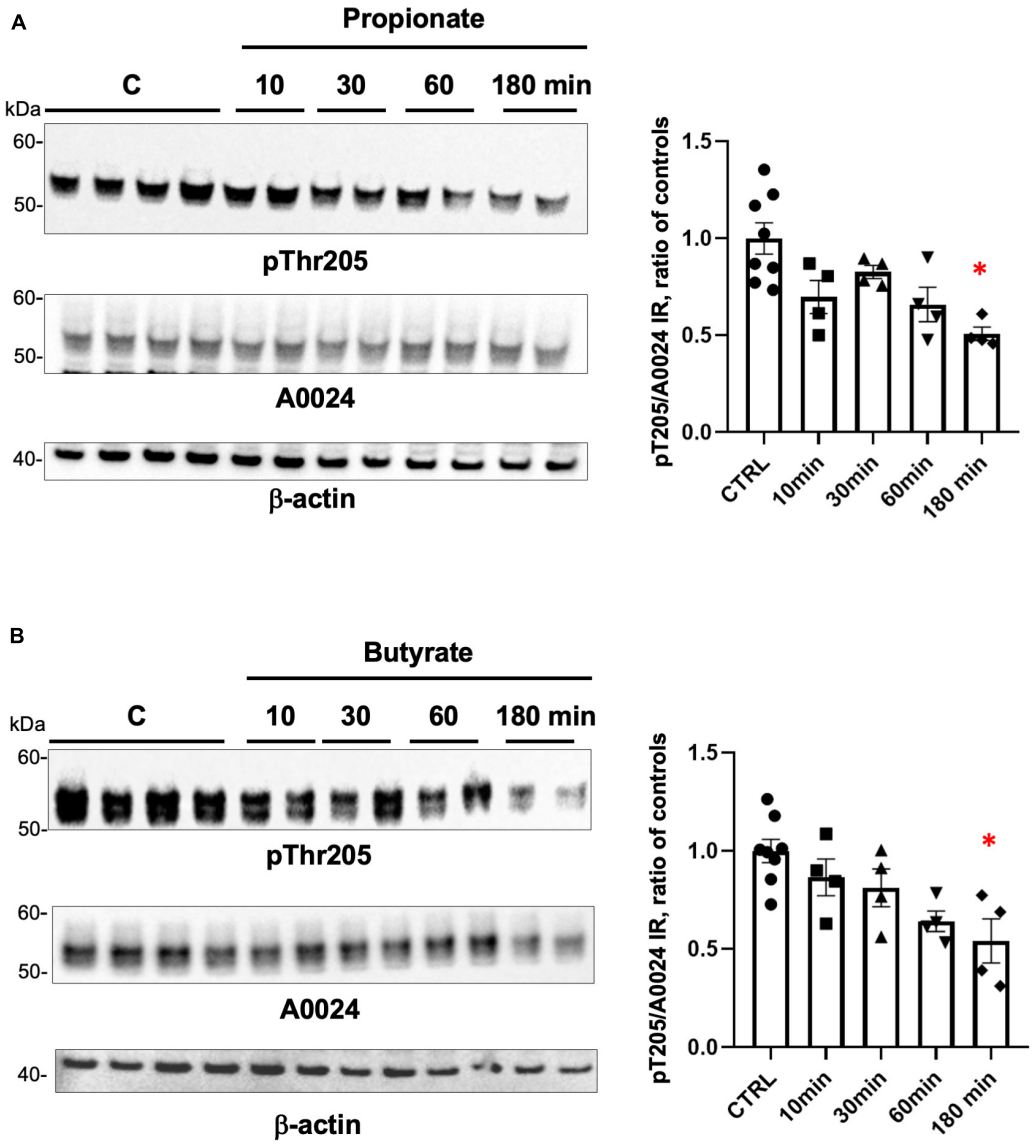

FIGURE 4
Regulation of tau phosphorylation by SCFA in GLUTag cells. **(A)** GLUTag cells were treated with 1 mM propionate for the indicated periods of time. Cell lysates were subjected to immunoblot analysis using antibodies against tau phosphorylated at Thr205 (pThr205) and total tau (A0024). Membranes were also probed with an anti-β-actin antibody to ensure equal protein loading. pThr205 immunoreactive bands were measured, normalized to the optical densities of total tau (A0024), and expressed as ratio of controls. Data correspond to mean ± SEM (n = 4–8, n indicates the number of wells; *$p < 0.05$, 180 min-treated vs control). **(B)** GLUTag cells were treated with 1 mM butyrate for the indicated periods of time. Cell lysates were analyzed as in panel **(A)** and quantification was performed as in panel **(A)**. Data correspond to mean ± SEM (n = 4–8, n indicates the number of wells; *$p < 0.05$, 180 min-treated vs. control).

reported that 0N, 1N and 2N isoforms are primarily localized to the cell bodies, nucleus, and axons, respectively (Liu and Götz, 2013), thereby suggesting that tau in EEC lines is primarily cytoplasmic. In order to properly compare the isoform profile of tau between human adult brain and EEC from human adult colon, it would have been useful to study in more detail tau isoforms in human EEC. We were however unable to do so for two main reasons: first, apart from 3R and 4R antibodies, other specific antibodies for 0N, 1N, and 2N isoforms did not work well for immunohistochemistry on paraffin sections, at least in our hands; second, in contrast to our previous research on the ENS, the use of frozen colonic sections for Western blot analysis was not possible because of the sparse distribution of EEC along the intestinal epithelium (Lionnet et al., 2018). Again, this precise characterization could benefit from laser microdissection approaches (Blatt and Srinivasan, 2008).

The phosphorylation of tau at multiple serine and threonine sites is the predominant mechanism by which tau functions are regulated (Guo et al., 2017). Dephosphorylation of tau from EEC lines produced a downward shift demonstrating that EEC-tau is, like CNS tau, phosphorylated (Hanger et al., 2002). Using a phospho-specific antibody, we further showed that tau was phosphorylated at Thr205 in GLUTag cells under basal condition and that phosphorylation at this residue was regulated by SCFA. What can be the role of tau and the consequences

of tau phosphorylation from a functional point of view? In this regard, it is tempting to compare our findings to those obtained in pancreatic β-cells. EEC and pancreatic β-cells share similar pathways of differentiation during embryonic development (Ryu et al., 2018). Remarkably, like EEC, pancreatic β-cells, also express tau (Maj et al., 2016; Wijesekara et al., 2018) and several studies showed that tau is critically involved in pancreatic β-cells function, insulin secretion and glucose homeostasis (Maj et al., 2016; Wijesekara et al., 2018). One might therefore suggest that, EEC-tau similarly regulates the secretion of peptide-hormones and that such a regulation is mediated via tau phosphorylation. Further experiments performed after silencing tau in EEC will be needed to answer this question.

Conclusion

In conclusion, we have characterized tau in the human colon and in EEC lines and we show that EEC tau phosphorylation can be regulated by SCFA. The data we have acquired on tau in EEC strongly supports additional future studies aimed at expanding our knowledge of peripheral pathology in tauopathies and at deciphering the physiological role of tau in CEE.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Fédération des Biothèques of the University Hospital of Nantes, according to the guidelines of the French Ethics Committee for Research on Humans and registered under the no. DC-2008-402. The patients/participants provided their written informed consent to participate in this study.

Author contributions

GC, NB, BC, IG, PC, and TO performed the experiments. MR-D managed the tissue sampling and biobanking. MR-D, GL, and PD supervised the study and wrote the final version of the manuscript. MN and HB provided critical feedback and helped shape the research. PD wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1166848/full#supplementary-material

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VIII. FICHE DE SYNTHÈSE

1. Résumé des travaux

Mes travaux depuis le doctorat ont porté sur 3 thématiques principales :

- Photopériode et fonction gonadotrope à l'UMR CNRS 6026 à l'Université de Rennes (Dir O. Kah) [Postdoc]
- Hémato-et immuno-toxicologie des mycotoxines puis étude du stress fongique au LBEM de l'Université de Brest (Dir G. Barbier) [Maître de Conférences]
- Nutrition périnatale et axe intestin-cerveau à l'UMR PhAN INRAE-Nantes Université (Dir H. Blottière) [Chargée de Recherche]

Le développement de mes activités de co-encadrement de doctorants ainsi que la consolidation d'une thématique dans la durée ont démarré avec mon recrutement à INRAE (2010). Mes travaux à l'UMR PhAN s'inscrivent dans le concept de l'origine développementale de la santé et des maladies (DOHaD) et principalement sur les effets de la nutrition périnatale sur le risque métabolique et le comportement alimentaire de la descendance, dans des modèles pré-cliniques chez le rat. La transmission de ces risques d'une génération à l'autre fait de la DOHaD un enjeu de santé publique majeur.

Mes travaux ont porté sur

1. La programmation métabolique du côlon dans un modèle de retard de croissance intra-utérin par restriction protéique maternelle.

Nous avons montré que le tissu adipeux viscéral qui s'installe à l'âge adulte dans ce modèle altérait l'intégrité de l'épithélium colique, pouvant contribuer aux désordres métaboliques observés chez les enfants nés avec un retard de croissance intra-utérin (co-encadrement de V. Haure-Mirande, 2 publications associées).

2. L'impact de la nutrition périnatale et du microbiote en début de vie sur l'axe intestin-cerveau, la fonction endocrine intestinale et le comportement alimentaire adulte.

Ces travaux ont montré que la restriction protéique maternelle diminuait la sensibilité vagale à la cholécystokinine, hormone satiétogène produite par les cellules entéro-endocrines, dérégulant la prise alimentaire de la descendance dans ce modèle. Les effets stimulants de la dénutrition périnatale sur la densité et la fonction de sensing nutritionnel de ces cellules étaient en discordance avec le retard de satiété, ce qui sous-tend une insuffisance de l'adaptation et/ou l'implication d'autres mécanismes (co-encadrement de M. Ndjim, 2 publications associées). Une modulation du microbiote néonatal par une supplémentation en oligosides prébiotiques stimulait la densité des cellules entéro-endocrines de type L et la production des peptides gastrointestinaux associés (GLP-1 et PYY) mais uniquement pendant la supplémentation et sans effet sur le comportement alimentaire à l'âge adulte. Un transfert de microbiotes maternels issus de mères minces ou de mères obèses dès la naissance à des ratons avait au contraire des effets sur le comportement alimentaire plus tard dans la vie sans que cela puisse être associé à une différence de composition du microbiote adulte (co-encadrement A.L. Pocheron, 2 publications associées). Le microbiote précoce aurait donc eu des effets sur l'axe intestin-cerveau en cours du développement postnatal, et des recherches de corrélations entre la composition et l'activité du microbiote et la transcriptomique intestinale sont en cours pour étayer des pistes mécanistiques.

3. L'effet de l'hyperglycémie *in utero* et de la lactation sur la fonction incrétine.

Dans un modèle de diabète gestationnel induit par un régime hypercalorique, mis en place dans le cadre de la thèse de P. Bobin (co-encadrement en cours), l'hyperglycémie pendant la gestation diminuait la densité des cellules entéro-endocrines de type L par rapport à la

population de cellules endocrines totales de façon sexe dépendante au sevrage. Ce résultat n'est pas en faveur de l'hypothèse d'un effet de la composition du lait sur cette composante endocrine intestinale puisque l'allaitement des ratons nés de mères hyperglycémiques pendant la gestation (DG) par des mères non DG ou maintenues sous régime hypercalorique pendant la lactation a le même effet. Néanmoins, des différences dans la composition en certains lipides bio-actifs du lait de femmes DG (cohorte DEPART) nous amènent à tester l'effet de ces composés *in vitro* sur des lignées de CEE humaines (et, en collaboration, sur les lignées de cellules ß-pancréatiques). Une supplémentation du lait maternel ou des formules infantiles en l'un de ces composés pourrait avoir des effets bénéfiques sur le risque métabolique des enfants nés de mères DG.

2. Résumé des projets

Mes projets pour les mois (et années) s'inscrivent dans la continuité des travaux précédents :

2.1. Les effets des métabolites bactériens sur la plasticité des CEE-L dans nos modèles de programmation

Ce projet vise à interroger les effets d'une modulation de la composition et de l'activité du microbiote intestinal sur la plasticité des CEE matures, capables de produire des peptides différents selon leur localisation dans le tube digestif. Ce concept récent pourrait expliquer les effets des oligosides prébiotiques sur la densité des cellules-L que nous avons observé dans l'iléon mais pas dans le côlon. Pour mener cette étude, nous avons idéalement besoins d'organoïdes afin de tester les effets de métabolites bactériens sur la prolifération et la différenciation des CEE. La mise au point chez le rat ayant échoué, nous réaliserons ce travail sur des organoïdes d'intestin de souris.

2.2 L'impact du microbiote néonatal sur le neurodéveloppement avec pour cible d'étude les interactions entre métabolites bactériens et les CEE

Ce projet s'inscrit dans l'étude des mécanismes visant à comprendre comment le microbiote précoce peut moduler le système endocrine et le neurodéveloppement des circuits impliqués dans la régulation du comportement alimentaire. Des souches de bactéries candidates identifiées dans notre de modèle de transfert de microbiote maternel « obésogène » seront cultivées et leurs métabolites seront testés sur de co-cultures de CEE et de neurones et glie entériques (collaboration avec l'UMR 1235 INSERM-NU Tens) et/ou d'afférences vagales. L'activation des CEE et des neurones en réponse aux métabolites Des marqueurs spécifiques des CEE et de l'activation neuronale/gliale seront utilisés.

Un nouveau projet collaboratif et financé (ANR Enteroendopark) sera développé :

2.3 Le rôle de la nutrition périnatale et du microbiote précoce sur le risque de Tauopathies plus tard dans la vie.

Ce projet fait suite à une collaboration avec l'UMR 1235 INSERM-NU Tens (P. Derkinderen) qui a débouché sur des résultats tout à fait originaux montrant l'expression et la phosphorylation de la protéine Tau dans les CEE. Nous souhaitons poursuivre la caractérisation et comprendre le rôle de Tau dans ces cellules épithéliales (non-neuronales) et interroger son implication dans les maladies neurodégénératives de l'axe intestin-cerveau (maladie d'Alzheimer, maladie de Parkinson). A partir des différents modèles de programmation par la nutrition périnatale et/ou la modulation du microbiote intestinal, nous analyserons l'expression de Tau et sa phosphorylation sur les prélèvements intestinaux disponibles à différents stades de vie. Des

approches in vitro seront menées sur des lignées de CEE transfectées ou non par des formes mutées de Tau afin de mesure les effets des métabolites bactériens identifiés dans nos modèles de programmation. Les résultats pourront par la suite être validés dans des modèles murins de tauopathies.

3. Synthèse des encadrements et co-encadrements

Encadrements et co-encadrements d'étudiants en thèse et Master2

	Nombre	Publications associées	Communications associées
Thèse	3 1 en cours	6	23 4 (thèse en cours)
Master2	11 (+1 en cours	2	8

Publications et communications associées à l'activité d'encadrement

Doctorants	Année soutenance	% co-encadrement	Publications associées	Communications associées
V. Haure-Mirande	2013	50	#6 et #9	#PO56, #PO54, #PO53, #P52, #P47, #PO46, #P45, #P43, #P42, #P41, #PO40, #P39, #PO30
M. Ndjim	2017	60	#5 et #7	#PO26, #P25, #P22, #P20, #P19, #P18
A.L. Pocheron	2019	50	#3 et #4 Revue #32	#PO17, #PO16, #P15, #P14, #PO13, #PO12, #P9, #P7, #P6, #PO4
P. Bobin	-	40		#P5, #PO4, #P3, #PO1

Master 2	Année soutenance	% co-encadrement	Publications associées	Communications associées
T. Le Calvez	2005	50	-	-
M. Fondrevez	2006	50	-	-
O. Habrylo	2007	100	#13	-
C. Poinsignon	2014	100	#7	#PO34, P36
A. Olier	2015	50	-	#P33, #P32, #P29, #P24
E. Gouyon	2016		-	#PO27
B. Houssais	2018	100	-	#PO4
M. Queignec	2020	50	#2	#P11, #PO10

S.K. Moussavi Seresht	2021	50	-	-
M. Michelland	2023	50	-	-
C. Bard	2023	100	-	-
C. Massias	2024	70		

Titre : Rôle de la nutrition périnatale et du microbiote sur l'axe intestin-cerveau, la fonction endocrine intestinale et le comportement alimentaire

Mots clés : Nutrition périnatale, programmation nutritionnelle, cellules entéro-endocrines, microbiote, homéostasie énergétique

Résumé : Selon le concept de l'origine développementale de la santé et des maladies (DOHaD), un environnement périnatal altéré, notamment nutritionnel, prédispose aux maladies métaboliques à l'âge adulte (diabètes, obésité, etc.). Ce document d'HDR synthétise les travaux réalisés autour des effets de la nutrition périnatale et du microbiote en début de vie sur la transmission du risque métabolique à la descendance, incluant les défauts de comportement alimentaire. La cible d'étude est l'axe intestin-cerveau, et notamment les cellules entéro-endocrines, sentinelles intestinales du système nerveux central.
Des manipulations nutritionnelles des mères en période périnatale ainsi que des modulations du microbiote précoce menées sur des rongeurs ont montré des défauts de régulation de prise alimentaire associés à une perte de sensibilité vagale et à des adaptations du sytème endocrine intestinal. Des pistes mécanistiques sont à l'étude afin de comprendre les interactions précoces entre le microbiote intestinal, les cellules entéro-endocrines et les neurones entériques/vagaux afférents. Les cellules entéro-endocrines (fonction incrétine) sont également étudiées dans un modèle d'hyperglycémie maternelle et de transmission de risque métabolique à la descendance. Enfin, un nouveau projet sur l'origine développementale des maladies neurodégénératives est en cours suite à la mise en évidence de la protéine Tau dans ces cellules.

Title: Perinatal nutrition and early microbiota on the gut-brain axis, entero-endocrine function and eating behavior

Keywords: Perinatal nutrition, metabolic programming, entero-endocrine cells, microbiota, homeostatic regulation

Abstract: According to the concept of the Developmental Origin of Health and Disease (DOHaD), an altered perinatal environment, such as malnutrition, predisposes to metabolic diseases later in life (diabetes, obesity, etc.). This HDR manuscript summarizes the work carried out on the effects of perinatal nutrition and microbiota in early life on the transmission of metabolic risk to offspring, including alteration of eating behavior. The main target is the gut-brain axis, particularly entero-endocrine cells, which are gut sentinels of the central nervous system.
Nutritional manipulations of mothers in the perinatal period and modulations of the microbiota in early life on rodents have shown defects in the regulation of food intake associated with a loss of vagal sensitivity and adaptations of the intestinal endocrine system. Mechanistic ways are being explored to understand the early interactions between the intestinal microbiota, entero-endocrine cells and enteric/vagal afferent neurons. Entero-endocrine cells (incretin function) are also being studied in a model of maternal hyperglycemia and the related metabolic risk in offspring. Finally, a new project on the developmental origin of neurodegenerative diseases is underway following the identification of the Tau protein in these cells.