



HAL
open science

Décryptage du rôle du microbiote intestinal dans le développement et la fonction des neurones neuropeptidergiques : Implications pour les troubles du neurodéveloppement.

Cassandre Morel

► **To cite this version:**

Cassandre Morel. Décryptage du rôle du microbiote intestinal dans le développement et la fonction des neurones neuropeptidergiques : Implications pour les troubles du neurodéveloppement.. Neurobiology. Normandie Université, 2023. English. NNT : 2023NORMR106 . tel-04510902

HAL Id: tel-04510902

<https://theses.hal.science/tel-04510902>

Submitted on 19 Mar 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Normandie Université



THÈSE

Pour obtenir le diplôme de doctorat

Spécialité **ASPECTS MOLECULAIRES ET CELLULAIRES DE LA BIOLOGIE**

Préparée au sein de l'**Université de Rouen Normandie**

Unravelling the role of gut microbiota in the development and function of hypothalamic neuropeptidergic neurons: Implications for neurodevelopmental disorders.

Présentée et soutenue par

CASSANDRE MOREL

Thèse soutenue le 20/10/2023

devant le jury composé de :

M. GERARD CLARKE	Professeur des Universités - Université National d'Irlande (IRL)	Rapporteur du jury
MME SYLVIE RABOT	Chargé de Recherche - UNIVERSITE PARIS-SACLAY	Rapporteur du jury
MME MURIEL DARNAUDERY	Professeur des Universités - UNIVERSITE DE BORDEAUX	Membre du jury
M. SERGUEI FETISSOV	Professeur des Universités - Université de Rouen Normandie	Président du jury
M. NICOLAS CHARTREL	Directeur de Recherche - Université de Rouen Normandie	Directeur de thèse
MME ROCHELLYS DIAZ HEIJTZ	Professeur Associé - Institut Karolinska de Stockholm (SUEDE)	Co-directeur de thèse

Thèse dirigée par **NICOLAS CHARTREL** (Neuroendocrine Endocrine and Germline Differentiation and Communication) et **ROCHELLYS DIAZ HEIJTZ** (Diaz Heijtz laboratory)

“We are all of us walking communities of bacteria. The world shimmers, a pointillist landscape made of tiny living beings.”

— Lynn Margulis.

Acknowledgments

Firstly, I would like to express my gratitude to all the members of my PhD defense jury, **Pr. Gerard Clarke, Dr. Sylvie Rabot, Pr. Muriel Darnaudéry** and **Pr. Sergueï Fetissov**, to come all the way to Rouen for the evaluation of this four-year work, and to allow me to discuss with such leading experts in the Microbiota-Gut-Brain axis field. I thank **Gérard Clarke** and **Sylvie Rabot** for kindly accepting to be the principal examiners of my thesis work and for providing me with their valuable expertise. I am particularly grateful for **Gerard Clarke's** presence as he allowed me to discover this fascinating research topic in the first place, during my 1st-year master's internship in his group in Cork. In addition, I am very grateful for the presence of **Muriel Darnaudéry**, especially for all her kind and useful advice during the PhD follow-up committee sessions. In the same direction, I thank **Sergueï Fetissov** for his numerous pieces of advice and his great interest for my PhD project.

Importantly, I would like to thank my PhD supervisors, **Dr. Rochellys Diaz Heijtz** and **Dr. Nicolas Chartrel** for their supervision and invaluable guidance. Thank you both for your patience and availability, and for helping me to be the scientist I am today. Particularly, I am very grateful for **Rochellys** to have shared all your proficient knowledge and expertise all along my PhD journey. It was a great chance to work and study in the Karolinska Institutet. Also, thank you for being available when I needed advice and support for our experiments, brainstorming and transmitting your analytical and scientific methods. Similarly, a warm thank you goes to **Nicolas**, for accompanying me either remotely and directly in France, and to have done everything to welcome me the best in the RegPep team when I arrived in Rouen in 2021. Above all, I am immensely grateful for your kindness and continuous emotional and professional support during my PhD, in particular during the redaction of this thesis as well as for our publication.

Likewise, I would like to thank the funding organizations of my PhD project, including the **Région Normandie** and the **Scientific Council for Medicine and Health** in Sweden (R. Diaz Heijtz), for allowing me to successfully complete my work. I am also grateful for the **University of Rouen**, the **INSERM organization** as well as the **Institut français de Suède** for enabling me to participate to prestigious international conferences and facilitating my travels between France and Sweden, notably.

I am also thankful for the former director, **Dr. Youssef Anouar**, and present directors and vice-directors of the NordiC Unit U1239, **Pr. Hervé Lefebvre** and **Dr. Jérôme Leprince**, for welcoming me in their unit during my PhD, and enabling me to perform this thesis in the best conditions possible. Notably, I would like to thank all the **members of the NordiC Unit** for their availability and kindness during the last two years of my PhD. I also thank **Marjorie Gras** for supporting me during my teaching time. A very special thanks goes to the whole **RegPep team**: firstly, the technicians **Yamina Cherifi** and **Saloua Takhlidjt** for their precious help and support during my experiments. I would like to thank warmly **Marie Picot** for her kindness and her trust, especially during my teaching time at the University of Rouen. In the same way, I thank **Dr. Carole Rovère**, that enabled me to meet my PhD supervisors and

start this project in the first place, as well as the other members of my PhD follow-up committee. Additionally, I am grateful for the help and valuable contribution from the PRIMACEN platform in our research building, especially the BIC Microscopy/Imaging service, including **Dr. Ludovic Galas** and **Damien Schapman**; as well as from the SRB animal facility, **Dr. Arnaud Arabo** and **Julie Maucotel** for their availability and flexibility.

Of course, I will never thank enough my dearest friends and teammates in KI, for all the moments we shared together in the lab and outside: **Ayoze Gonzalez Santana**, **Julia Spielbauer**, **Inés Martinez Sanchez**, **Marie Armbruster**, **Léna Schleich** and **Hayatte Dounia Mir**. **Ayoze**, “my guardian angel”, there are not enough words to describe my gratitude to you, especially for all the help in my project and your unconditional emotional support. **Inés**, thank you for all your kindness, patience and precious help during the preparation of the paper, especially. **Julia**, **Léna**, **Marie** and **Hayatte**, I am lucky to have shared, worked, laughed, and discovered Sweden with you, none of this adventure would have been possible without you.

Désormais en français, j’aimerais remercier tout particulièrement ma famille et mes chers amis francophones. Tout d’abord, merci à mes colocataires et amis du 73 qui sont un immense soutien au quotidien. A **Clara**, ma « sœur de thèse » et **Baptiste**, qui m’ont non seulement nourrie, mais aussi soutenue de bout en bout. Également, un très grand merci aux Losay « Meuh », **Thomas** et **Matthieu**, à **Blandine**, et évidemment à mes chers **Jean-Louis** et **Margaux**. *Last but certainly not least*, à **Vincent**, merci pour tout, mais aussi à **Thomas** et **Marat**, qui tous trois apporté un tournant significatif avec LT, j’ai hâte de vivre la suite! Je remercie également mes anciens colocataires rouennais, **Ahmed** et **Adèle**, avec lesquels nous avons tant partagé pendant ma première année à Rouen.

Un très grand merci à mes amies de longue date, **Noémie**, **Pauline**, **Carole**, **Hadjer** et **Clémence**, qui, même de loin, ont été présentes et importantes lors de cette aventure. De même, j’adresse un grand merci à mon *alter ego* irlandaise, **Cassandra**. Merci également à mon logeur et ami, **Thierry**, pour avoir été à mes côtés lors des bons et mauvais moments, et pour avoir partagé un peu du pays à Kungsholmen.

Enfin, mes plus grands remerciements et mes pensées les plus affectueuses vont à ma famille et surtout mes parents, mes sœurs, mon oncle **Franck** et ma tante **Anne** et à mes chers grands-parents, dont je suis honorée « de faire la fierté ». **Mon père, ma mère et mes sœurs, Margaux et Ysée**, mes piliers, je pense ne pas pouvoir exprimer toute la gratitude que je vous dois, surtout pour votre soutien, pour avoir été là dans les moments les plus difficiles et de m’avoir aidée à garder le cap, merci du fond du cœur.

Table of contents

ABSTRACT	3
RESUME	4
SCIENTIFIC OUTPUT	5
TEACHING & SUPERVISING ACTIVITIES	6
LIST OF ABBREVIATIONS	7
LIST OF FIGURES AND TABLES	9
GENERAL INTRODUCTION	12
1 THE GUT MICROBIOTA AND HOST HEALTH	12
1.1 THE GUT MICROBIOTA AS A MICROBIAL ORGAN	12
1.1.1 DEFINITIONS	12
1.1.2 THE HUMAN GUT MICROBIOTA	13
1.2 THE GUT MICROBIOTA AND ITS INTERACTIONS WITH HOST IMMUNITY AND METABOLISM	15
1.2.1 NUTRITIONAL, DIGESTIVE, AND METABOLIC ROLES	15
1.2.2 ROLE OF MICROBIOTA IN THE GI TRACT DEVELOPMENT AND FUNCTION	18
1.2.3 ROLE OF MICROBIOTA IN THE EDUCATION AND MAINTENANCE OF THE IMMUNE SYSTEM	20
1.3 THE MICROBIOTA-GUT-BRAIN AXIS	22
1.4 BIOLOGICAL SIGNALING PATHWAYS IN THE MICROBIOTA-GUT-BRAIN AXIS	25
1.4.1 NEURONAL PATHWAYS	27
1.4.2 ENTEROENDOCRINE SIGNALING	28
1.4.3 NEUROENDOCRINE PATHWAY – COMMUNICATION WITH THE HPA AXIS	28
1.4.4 NEUROIMMUNE AND IMMUNE PATHWAYS	29
1.4.5 MICROBIAL METABOLITES SIGNALING	31
2 THE MICROBIAL COLONIZATION PROCESSES AND THE DEVELOPING BRAIN	33
2.1 ASSEMBLY AND MATURATION OF THE INFANT GUT MICROBIOTA	33
2.1.1 MICROBIAL TRANSMISSION, COLONIZATION, AND SUCCESSION	33
2.1.2 IMPLICATIONS FOR THE DEVELOPING GI TRACT, IMMUNE AND METABOLIC SYSTEMS	37
2.2 FACTORS INFLUENCING THE INFANT MICROBIOTA AND HEALTH	42
2.2.1 MODE OF DELIVERY	43
2.2.2 FEEDING MODE (BREASTFEEDING VS. FORMULA)	45
2.2.3 MATERNAL INFECTIONS AND STRESS	46
2.2.4 MATERNAL DIET	47
2.2.5 ANTIBIOTIC EXPOSURE	49
2.3 INFLUENCE OF THE GUT MICROBIOTA ON NEURODEVELOPMENTAL PROCESSES	52
2.3.1 NEUROGENESIS AND NEURONAL MIGRATION	53

2.3.2	SYNAPTIC DEVELOPMENT, DENDRITIC GROWTH AND MYELINATION	55
2.3.3	BLOOD BRAIN BARRIER INTEGRITY	57
2.3.4	MICROGLIA MATURATION	58
3	GUT MICROBES, NEUROPEPTIDES, AND SOCIO-EMOTIONAL BEHAVIORS	61
3.1	NEUROPEPTIDERGIC SYSTEMS REGULATING SOCIO-EMOTIONAL BEHAVIORS	61
3.1.1	DEVELOPMENT AND FUNCTION OF THE OXYTOCIN AND THE VASOPRESSIN SYSTEMS	61
3.1.2	REGULATION OF SOCIO-EMOTIONAL BEHAVIORS BY OXYTOCIN AND VASOPRESSIN	72
3.1.3	DEVELOPMENT OF THE CRH SYSTEM AND THE HPA AXIS	78
3.2	GUT MICROBES AND NEUROPEPTIDERGIC SYSTEMS	84
3.2.1	GUT MICROBES IN THE DEVELOPMENT OF THE OT/AVP SYSTEMS	84
3.2.2	GUT MICROBES IN THE DEVELOPMENT OF CRH AND THE HPA AXIS	85
3.3	ANTIBIOTIC EXPOSURE IN EARLY LIFE AND IMPLICATIONS FOR NEURODEVELOPMENTAL DISORDERS	88
3.3.1	ANTIBIOTIC EXPOSURE AND NEURODEVELOPMENTAL DISORDERS IN HUMANS	88
3.3.2	ANTIBIOTIC EXPOSURE AND NEURODEVELOPMENTAL DISORDERS IN RODENTS	91
	THESIS OBJECTIVES	102
	RESULTS – PUBLICATION	105
	RESULTS – PUBLICATION: SUPPLEMENTARY DATA	120
	GENERAL DISCUSSION & PERSPECTIVES	131
1.	POTENTIAL LINK BETWEEN KEY MICROBIAL SPECIES AND NEUROBEHAVIORAL ABNORMALITIES	131
2.	EMERGING MICROBIOTA-FOCUSED INTERVENTIONS TO TREAT NEURODEVELOPMENTAL DISORDERS	135
3.	BIOLOGICAL PATHWAYS IMPLICATED IN THE CROSSTALK BETWEEN THE GUT MICROBIOTA AND THE BRAIN: POTENTIAL IMPLICATIONS FOR EARLY LIFE ANTIBIOTIC EXPOSURE	140
4.	THE PEPTIDOGLYCAN SIGNALING PATHWAY IN THE DEVELOPMENT OF NEUROPEPTIDERGIC SYSTEMS: IMPLICATIONS FOR NEURODEVELOPMENTAL DISORDERS	142
5.	GENERAL CONCLUSION	147
	BIBLIOGRAPHY	149

Abstract

The gut microbiota – the trillions of microorganisms inhabiting the gastrointestinal tract, including bacteria, yeasts and viruses – is increasingly recognized as a key environmental factor that shapes host development and physiology. Over the last two decades, the gut microbiota has been identified as an essential component of the gut-brain axis, leading to the emerging concept of the "Microbiota-Gut-Brain axis". Preclinical studies using germ-free animals have demonstrated that the gut microbiota influences a wide range of neurodevelopmental processes including, the maturation and function of microglia, primary resident immune cells of the brain, blood brain barrier (BBB) integrity, and myelination. Moreover, several epidemiological studies suggest that broad-spectrum antibiotic exposure around the time of birth could have detrimental health outcomes, including childhood behavioral and emotional issues. However, it is unclear whether antibiotic exposure during a narrow perinatal window could influence neurodevelopmental outcomes. In this context, the aim of this thesis was to investigate the impact of maternal antibiotic exposure during a narrow critical perinatal window (*i.e.*, last week of pregnancy and first three postnatal days) on physiological and neurobehavioral outcomes of the offspring, including the development of socio-emotional behaviors and the expression of genes associated with intestinal barrier function, BBB integrity and the oxytocin and vasopressin receptor systems. Our results demonstrate that neonatal offspring from antibiotic-treated dams display an altered pattern of ultrasonic communication, which was more pronounced in males. Moreover, juvenile offspring from antibiotic-treated dams showed reduced social motivation and interaction, as well as context-dependent anxiety-like behavior. In addition, the juvenile offspring from exposed dams also displayed an altered gut microbiota composition. However, no changes were observed in locomotor or exploratory activity. This behavioral phenotype is strongly male-biased and is associated with BBB impairments and a mild colonic inflammatory response. Strikingly, perinatal ampicillin exposure leads to reduced gene expression of the oxytocin receptor in the prefrontal cortex of both male and female juvenile offspring and increased gene expression of the vasopressin receptor 1a in the amygdala of the female offspring, which are key regions involved in the regulation of socio-emotional behaviors. Altogether, these findings highlight the importance of the maternal microbiota in early-life, and how its perturbation by a widely used antibiotic could contribute to atypical social and emotional development of offspring in a sex-dependent manner.

Key words: Perinatal; Antibiotics; Gut microbiota; Socio-emotional behaviors; Neuropeptidergic systems.

Résumé

Le microbiote intestinal, ou les milliards de micro-organismes peuplant le tractus gastro-intestinal, y compris les bactéries, les levures et les virus, est considéré comme un facteur-clé pour le développement et la physiologie de l'hôte. Au cours des deux dernières décennies, le microbiote intestinal a été identifié comme un composant essentiel de l'axe intestin-cerveau, ce qui a conduit à l'émergence du concept d' « Axe Microbiote-Intestin-Cerveau ». Des études précliniques utilisant des animaux axéniques ont montré que le microbiote intestinal influence divers processus neurodéveloppementaux, tels que le développement et la fonction de la microglie, la formation de la barrière hémato-encéphalique (BHE) et la myélination. Plusieurs études épidémiologiques suggèrent que l'exposition à des antibiotiques à large spectre au moment de la naissance pourraient avoir des effets néfastes sur la santé, comme des problèmes comportementaux et émotionnels chez l'enfant. Néanmoins, les conséquences d'une exposition aux antibiotiques sur le neurodéveloppement pendant une période limitée autour de la naissance est aujourd'hui encore inconnu. Dans ce contexte, l'objectif de cette thèse est d'étudier les effets d'un traitement maternel à l'ampicilline pendant une fenêtre périnatale étroite (c'est-à-dire la dernière semaine de gestation et les trois premiers jours postnataux) sur la physiologie et le neuro-comportement de la progéniture en rapport avec les troubles du neurodéveloppement, y compris les comportements socio-émotionnels et l'expression de gènes associés à la fonction de la barrière intestinale, à l'intégrité de la BHE, et aux systèmes ocytocinergiques et vasopressinergiques. Nos résultats montrent que la progéniture issue de mères exposées aux antibiotiques présentent des déficiences dans la communication ultrasonique néonatale, de manière plus prononcée chez les mâles que chez les femelles. Par ailleurs, les petits issus de mères traitées aux antibiotiques montrent une diminution de leur comportement social, des altérations du comportement anxieux, ainsi que des modifications du microbiote intestinal au cours de la période juvénile. Cependant, aucun changement n'a été observé dans l'activité locomotrice ou exploratoire. Ce phénotype comportemental est fortement masculin et est associé à une altération de la BHE et à un léger état inflammatoire du côlon. L'exposition périnatale à l'ampicilline provoque également une réduction des niveaux d'expression génique du récepteur de l'ocytocine dans le cortex préfrontal, ainsi qu'une augmentation de l'expression génique du récepteur de la vasopressine 1a dans l'amygdale, seulement chez la femelle ; qui sont des régions clés impliquées dans la régulation des comportements socio-émotionnels. Dans l'ensemble, ces résultats soulignent l'importance du microbiote maternel au début de la vie et la façon dont sa perturbation par un antibiotique largement utilisé pourrait contribuer au développement social et émotionnel atypique de la progéniture, en fonction du sexe.

Mots clés: Antibiotiques ; Microbiote intestinal ; Période périnatale ; Comportements socio-émotionnels ; Systèmes neuropeptidergiques.

Scientific output

Published article. Morel C, Martinez Sanchez I, Cherifi Y, Chartrel N, Diaz Heijtz R. “Perturbation of maternal gut microbiota in mice during a critical perinatal window influences early neurobehavioral outcomes in offspring”. *Neuropharmacology*. May 2nd, 2023; 229:109479. doi: [10.1016/j.neuropharm.2023.109479](https://doi.org/10.1016/j.neuropharm.2023.109479)

Flash Talk/Poster communication. Morel C, Martinez Sanchez I, Cherifi Y, Chartrel N, Diaz Heijtz R. “Perturbation of maternal gut microbiota in mice during a critical perinatal window influences early neurobehavioral outcomes in offspring”. Presented at:

- The *45ème Colloque de la Société de Neuroendocrinologie*, September 27th-29th, 2023, in Rouen, France.

Poster communication. Morel C, Martinez Sanchez I, Cherifi Y, Chartrel N, Diaz Heijtz R. “Perturbation of maternal gut microbiota in mice during a critical perinatal window influences early neurobehavioral outcomes in offspring”. Presented at:

- The *Mind, Mood and Microbes Conference 2023*, May 10th-11th, 2023, in Amsterdam.
- The *Journées de l’Ecole Doctorale 2023*, April 6th-7th, 2023, in Rouen, France.

Poster communication. Morel C, Chartrel N, Diaz Heijtz R. “Bacterial peptidoglycan-sensing molecules are expressed in the mouse hypothalamus during specific temporal windows of postnatal development.” Presented at the:

- *7th Bordeaux Neurocampus Conference 2022*, May 18th-20th, 2022, in Bordeaux, France.
- *8th Mediterranean Neuroscience Society Conference*, May 29th-June 2nd, 2022, in Dubrovnik, Croatia.
- *Journée normande de la Recherche Biomédicale 2022*, June 6th, 2022, in Rouen, France.

Oral presentation. Morel C, Chartrel N, Diaz Heijtz R. “Bacterial peptidoglycan-sensing molecules are expressed in the mouse hypothalamus during specific temporal windows of postnatal development.”. Presented at the:

- *SNE Young Investigators Webinar*, March 24th, 2022, online.

Teaching & Supervising activities

Internship supervision. Diaz Heijtz Laboratory, Karolinska Institutet, Stockholm.

- **1st-year master's student** - Léna Schleich – April – July 2021

“Teaching mission” (Mission Enseignement 32h). Université de Rouen Normandie.

- **32h « Cell culture Techniques » Tutorials (TD) & Practicals (TP)** – 2nd-year Bachelor students – November-December 2021.

« One-off teaching » (Vacations). Université de Rouen Normandie.

- **4h « Genetics and Expression in Eukaryotic Genomes » Practicals** – 3rd-year Bachelor students – January-February 2022.
- **13h « Cellular functions » Practicals** – 3rd-year Bachelor students – March 2022.

« Half-time Teaching Contract » (Contrat Demi-ATER). Université de Rouen Normandie.

- **54h « Genetics and Expression in Eukaryotic Genomes » Practicals (TP)** – 3rd-year Bachelor students – January-February 2023.
- **36h « Induction of the Lactose Operon expression in *E. coli* »** – 1st-year Bachelor students - March 2023.
- **12h « Cellular functions » Practicals** – 3rd-year Bachelor students – March-April 2023.

List of abbreviations

4-EP 4-ethylphenol	BNST Bed nucleus of the stria terminalis	GALT Gut-associated lymphoid tissues
4-EPS 4-ethylphenyl sulfate	CC Cingulate cortex	GDNF Glial cell-line-derived neurotrophic factor
5-HT 5-hydroxytryptamine or Serotonin	CeA Central amygdala	GF Germ-free
ABX Antibiotics	CFU Colony-forming units	GI Gastrointestinal tract
ACTH Adrenocorticotrophic hormone	ClpB Caseinolytic protease B	GLP-1 Glucagon-like peptide 1
ADHD Attention-deficit/Hyperactivity disorder	CNS Central nervous system	GPR41 G protein-coupled receptor 41
ADPN Antero-dorsal preoptic nucleus	CNTF Ciliary neurotrophic factor	GPR43 G protein-coupled receptor 43
AhR Aryl hydrocarbon receptor	CPu Caudate putamen	GR Glucocorticoid receptor
AMPs Antimicrobial peptides	CRH Corticotropin-releasing hormone	HMO Human milk oligosaccharide
ANS Autonomic nervous system	CRHR1 CRH receptor 1	HNS Hypothalamo-neurohypophysial system
AON Anterior olfactory nucleus	CRHR2 CRH receptor 2	HPA Hypothalamic-pituitary-adrenals axis
ARC Arcuate nucleus	CSF Cerebrospinal fluid	HPC Hippocampus
ASD Autism spectrum disorder	DCs Dendritic cells	I3A Indole-3-aldehyde
AVP Vasopressin or Arginine-vasopressin	DG Dentate gyrus	IBD Inflammatory bowel disease
AVPR1a Vasopressin receptor 1a	DMH Dorsomedial hypothalamus	IDO Indoleamine 2,3-dioxygenase
AVPR1b Vasopressin receptor 1	DRN Dorsal raphe nucleus	IECs Intestinal epithelial cells
AVPR2 Vasopressin receptor 2	E Embryonic day	IFN Interferon
BAs Bile acids	EECs Enteroendocrine cells	IL Interleukin
BBB Blood brain barrier	ENS Enteric nervous system	ILC Innate lymphoid cells
BDNF Brain-derived neurotrophic factor	Erdr1 Erythroid differentiation regulator-1	
BLA Basolateral amygdala	FMT Fecal microbiota transplant	
	GABA γ -Amino butyric acid	

ILC3 Group 3 innate lymphoid cells	MR Mineralocorticoid receptor	PRR Pattern recognition receptors
ILF Intestinal lymphoid follicles	MS Multiple sclerosis	PSA Polysaccharide A
iNKT Invariant natural killer T cells	NAc Nucleus Accumbens	PSD-95 Postsynaptic density protein 95
IPA Indole-3-propionate	NDD Neurodevelopmental disorders	PV Periventricular nucleus of the thalamus
JAM Junction adhesion molecules	NO Nitregeric	PYY Peptide YY
KO Knockout	NOD Nod-like receptor	RA Retinoic acid
LA Lipotechoic acid	NTS Nucleus tractus solitarius	SCFAs Short-chain fatty acids
LC Locus coeruleus	OB Olfactory bulb	SCH Hypothalamic supraschiasmatic nucleus
LPS Lipopolysaccharide	OT olfactory tubercle	SFB Segmented filamentous bacteria
LS Lateral septum	OXT Oxytocin	sIgA Secreted immunoglobulin A
MAIT Mucosal-associated invariant T cells	OXTR Oxytocin receptor	SPF Specific pathogen-free
MAMP Microbe-associated molecular pattern	P Postnatal day	TGF-β Tumor growth factor β
MDP Muropeptide	PAG Periaqueductal gray	Th T helper cells
MeA Medial amygdala	PBN Parabrachial nucleus	TLR Toll-like receptor
MFGM Milk fat globule membrane	PepT1 Peptidoglycan transporter 1	TPH1 Tryptophan hydroxylase 1
MIA Maternal immune activation	PeVN Hypothalamic periventricular nucleus	Tregs Regulatory T cells
MLNs Mesenteric lymph nodes	PFC Prefrontal cortex	VTA Ventral tegmental area
MPOA Medial preoptic area	PGLYRP Peptidoglycan-recognition protein	ZO <i>Zonula occludens</i>
	PGN Peptidoglycan	
	PPi Posterior pituitary	

List of Figures and Tables

Figure 1. The six main bacterial phyla composing the mammalian gut microbiota and some examples of their respective taxonomic groups.	14
Figure 2. The interplay between the intestinal cell barrier, the mucosal immune system and the gut microbiota.	19
Figure 3. Pioneer studies on the development of the Microbiota-Gut-Brain axis and the relevance for neurodevelopmental disorders (NDD).	23
Figure 4. Potential signaling pathways of the Microbiota-Gut-Brain axis.	26
Figure 5. Parallel development of the microbiota, the mucosal immune system and the GI tract during the perinatal period, and factors influencing the developing gut microbiota.	37
Figure 6. Factors influencing the gut microbiota colonization process in the infant.	42
Figure 7. Beneficial effects of SCFAs derived from maternal high-fiber diet offspring obesity predisposition are mediated by embryonic SCFAs receptors.....	49
Figure 8. Brain developmental milestones in both mice and humans, and parallel gut colonization process.	52
Figure 9. Maternal microbial metabolites influence prenatal brain wiring and related sensorimotor behaviors.	54
Figure 10. Schematic representation of the hypothalamo-neurohypophyial system (HNS)....	62
Figure 11. Spatiotemporal development of OXT and AVP neurons in the mouse hypothalamus.	63
Figure 12. Maturation of OXT and AVP neurons electrophysiology and morphology. A.	65
Figure 13. Localization of OXT neurons, projections and OXTR expression in the rodent brain.	67
Figure 14. Localization of AVP neurons, projections and Avpr1a/Avpr1b expression in the rodent brain.	68
Figure 15. Behavioral and physiological functions of oxytocin and vasopressin in mammals.	73
Figure 16. Expression of the CRH receptors, CRHR1 and CRHR2, in the rodent brain.....	79

Table 1. Studies on early life antibiotic exposure and effects on physiology and neurobehavior in humans.....	89
Table 2. Studies on early life antibiotic exposure and effects on physiology and neurobehavior in rodents.....	92
Figure 17. Graphical abstract of the results presented in C. Morel et al., 2023 (Neuropharmacology).....	103

General Introduction

General Introduction

1 The gut microbiota and host health

1.1 The gut microbiota as a microbial organ

The existence of a connection between our inner organs, including the gut, and the nervous system has long been appreciated by humankind. No later than 2000 years ago, traditional Chinese doctors already acknowledged that the stomach and the spleen were affected by anxiety and negative moods ¹. In 1851, Joseph Leidy described, in remarkable detail, some microorganisms as a 'fauna and flora within living animals' that he named respectively 'entozoa' and 'entophyta', after microscopic observations of the intestinal tracts of centipedes and beetles ². From his pioneering advances in the field of Microbiology and Immunology, Pasteur was one of the first scientists in the 19th century, to acknowledge that microbes are not just pathogens, but indispensable for any form of life ³. These ancient assumptions thus suggested the potential interdependence between humans and microbes. Nonetheless, one of the first scientific evidence of a gut-brain crosstalk was brought in the 19th century, with an important observation of a U.S. Army doctor, Dr. William Beaumont, later recognized as the 'Father of Gastric Physiology'. His wounded patient, Alexis St. Martin, was left with an opening in the stomach following surgery, which allowed Beaumont to observe a significant alteration of digestion rate when the patient became angry or irritable ^{4,5}. Thus, the concept of a complex bidirectional communication between the gastrointestinal (GI) tract and the central nervous system (CNS), referred to as 'the gut-brain axis', is not new historically, but modern research is still working hard to unlock its secrets.

1.1.1 Definitions

The microbiota refers to the collection of microorganisms residing within and on us, as they are found on various body sites including the skin, the airways, the genitalia and the GI tract ⁶⁻⁸. The microbiota includes fungi, archaea, protozoa, viruses, but bacteria are the most represented microorganisms in that complex ecosystem. The human microbiota is composed of trillions of microorganisms and represents an estimated ratio of microbial to human cells of about 1 to 1.3 (for a 70 kg "reference man") ⁹. If our commensal microbes are not outnumbering our own cells as previously believed, the microbiome, or the collection of genes within the human microbiota, counts about 2-20 million genes, exceeding the human genome by at least a factor of 100 ⁸⁻¹⁰. Many of these bacterial genes encode enzymes that perform metabolic functions, not achievable

by the host, and produce metabolites and other microbial metabolic by-products that can impact host health and disease ^{11–13}.

1.1.2 The human gut microbiota

The human GI tract is by far the most densely colonized organ, along which gut microbes are heterogeneously distributed. The stomach has a relatively low microbial density with 10 colony-forming units (CFU)/gram, and this concentration increases progressively along the GI tract. The most important density is found in the ileum (10^7 CFU/g) and the colon (10^{12} CFU/g), where 70% of all the human gut microbes are located ^{6,14}. As the GI tract is mostly oxygen-free (*e.g.*, in anaerobic conditions), it harbors a majority of anaerobic microorganisms, but also counts facultative anaerobes and aerobic microorganisms ^{14,15}. However, due to the limitation of culture-based methods to identify anaerobic microbes before the 1990's, a large part of the gut microbiota remained unidentified until the advent of sequence-based methods and bioinformatic tools. Thanks to these more advanced methods, the human microbiota composition was further characterized, through independent cohort studies ^{6,7}, and later, with larger population-based research initiatives, such as the Human Microbiome Project Consortium ^{8,10}. Together, these efforts enabled the description of the majority of the human gut microbiota, which is completed with improving multi-omics methods ¹⁶. Nearly 2776 prokaryotic species inhabit our gut and were classified into 12 different bacterial phyla. The *Bacteroidetes* and the *Firmicutes* phyla represent, for instance, 90% of the total human gut microbiota. The rest of the detected populations consist of the *Proteobacteria*, the *Actinobacteria*, the *Fusobacteria* and the *Verrucomicrobia* phyla ^{10,12,16}. Some examples of gut microbiota taxonomic groups are given in the figure below (see **Figure 1**).

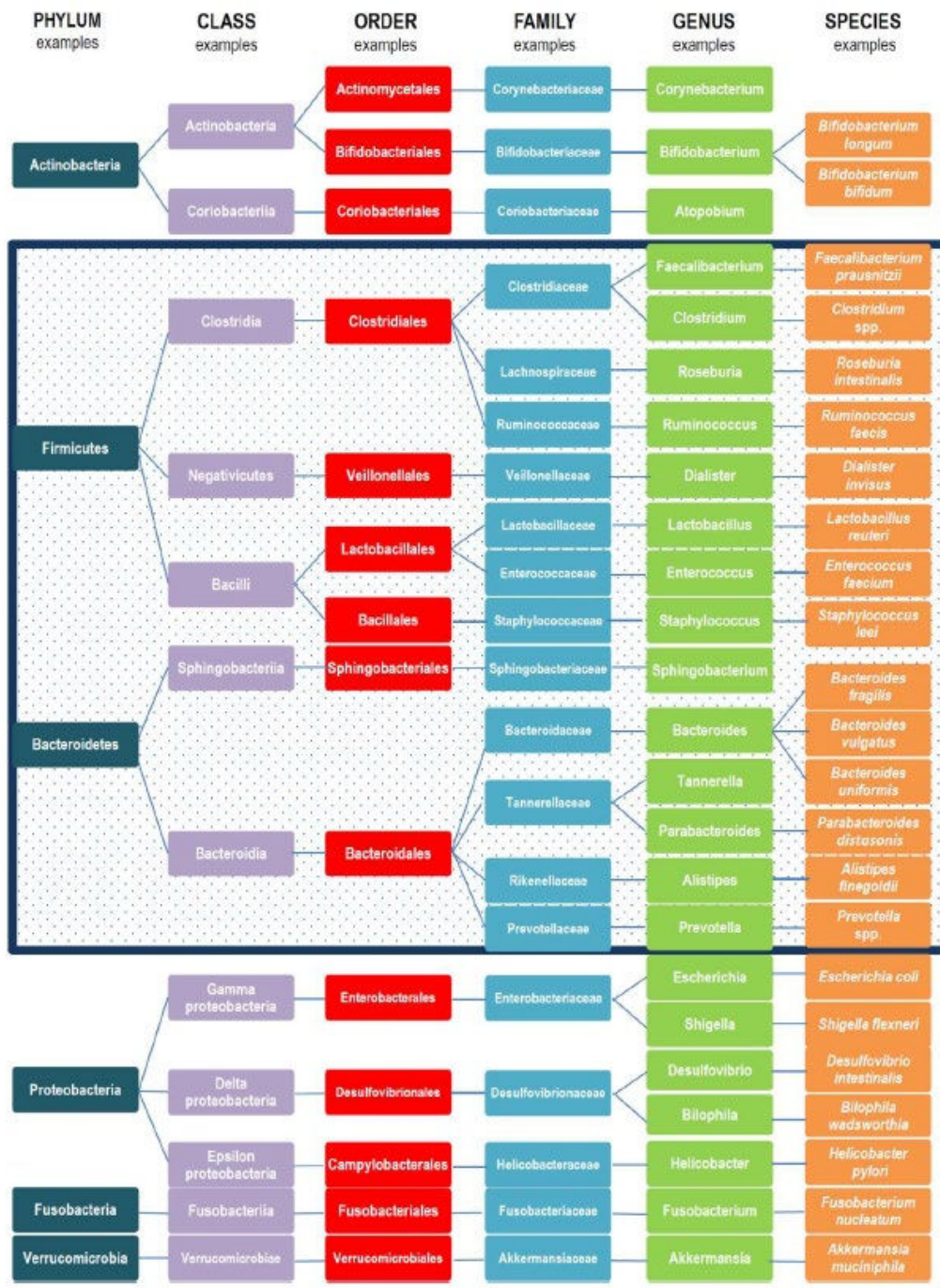


Figure 1. The six main bacterial phyla composing the mammalian gut microbiota and some examples of their respective taxonomic groups. The blue box comprises some examples of bacterial taxa belonging to the *Bacteroidetes* and *Firmicutes* phyla, that account for 90% of all the gut microbiota composition. Of note, the bacterial names presented here belong to a former nomenclature, as taxonomic nomenclature of prokaryotes has been revised recently¹⁷. Figure from *Rinninella et al.* (2019)¹².

Moreover, microbial numbers and diversity in the gut change inter- and intra-individually, but it has been acknowledged that a healthy gut microbiota is overall stable over time^{12,18}. This can

be explained especially by the fact that although the gut microbiota composition is unique at the species level, there is a pressure to maintain similar proportions of bacterial phyla (*i.e.*, the *Bacteroidetes/Firmicutes* ratio), which suggests a functional redundancy of the gut microbiota. In fact, we know now that the gut microbiota is resilient to changes after various disturbances and always maintains a dynamic equilibrium, that is beneficial for host health¹⁸. Furthermore, various factors such as diet, age, host genetics, exposure to medication or infections directly influence the gut microbiota¹² but might disturb this healthy equilibrium. In turn, this can lead to another equilibrium detrimental for host health, whereby the gut microbiota contributes to the risk or the progress of diseases. This perturbed gut microbiota state is referred to as ‘dysbiosis’.

1.2 The gut microbiota and its interactions with host immunity and metabolism

As mentioned above, the gut microbiome contains at least 100 times more genes than the human genome and thus accounts for a major part in host homeostasis and overall health. As such, it is not surprising that gut commensals are implicated in a number of physiological processes in host periphery, including the development and function of the GI and immune systems, as well as the regulation of host metabolism^{12,13,19}.

1.2.1 Nutritional, digestive, and metabolic roles

It is now well documented that gut commensals regulate many metabolic pathways and energy homeostasis in the host. The gut microbiome provides a set of genes that enables the host to extract otherwise inaccessible nutrients from the diet, such as polysaccharides (*i.e.*, complex sugars), proteins, polyphenols and contributes to the synthesis of essential vitamins and bioactive metabolites¹³.

Synthesis of essential vitamins. The gut microbiota is involved in the synthesis of essential vitamins as it synthesizes K and B vitamins, that cannot be synthesized by the host itself. A pivotal study in the GF rat showed for instance that absence of microorganisms leads to blood clotting deficiency, a physiological process in which vitamin K is required²⁰. Moreover, *Magnusdottir et al.*²¹ showed that a quarter of the eight essential B vitamins daily requirements are synthesized by the gut microbiota.

Generation of phenols and indoles and intestinal health. The gut microbiota participates to the generation of other bioactive by-products such as phenols and indoles, amines and sulfurous

compounds from amino acid fermentation, including the fermentation of aromatic amino acids (*i.e.*, tryptophan, phenylalanine and tyrosine) ²². Indoles and its metabolic by-products derive from the essential amino acid tryptophan. Some intestinal microbes, such as tryptophanase-expressing bacteria *Clostridium sporogenes* and *Lactobacillus spp.*, are able to catabolize tryptophan into indole, indole-3-propionate (IPA) and indole-3-aldehyde (I3A), respectively ^{23,24}. They are produced mainly from *Bacteroides* and *Enterobacteriaceae* species ²². They have significant physiological effects in the gut and extraintestinal organs via the binding to the aryl hydrocarbon receptor (AhR). Indole and I3A, for instance, participate in the reinforcement of the intestinal barrier by acting on the tight junction protein expression, the activation of glucagon-like peptide 1 (GLP-1)-producing-enteroendocrine cells to regulate appetite, and the modulation of gut mucosal immune cells. However, indole is converted into indoxyl sulfate by the host liver, that, in elevated levels, can be detrimental to host health ²².

Production of neurotransmitters. Most importantly, bacteria can catabolize amino acids into neurotransmitters. Microbial conversion of tryptophan leads to significant amounts of serotonin (5-HT), which is a crucial neurotransmitter involved in both the enteric nervous system (ENS) and CNS functioning. Gut microbes influence, directly and indirectly, the availability of tryptophan and the development of a normal serotonergic system throughout life and influences 5-HT biosynthesis by upregulating the tryptophan hydroxylase 1 (Tph1) expression in colonic enterochromaffin cells ^{25,26}. In addition to the 5-HT pathway, tryptophan is also metabolized by the host into kynurenine and its metabolites, kynurenic and quinolinic acids, along the kynurenine pathway. Both kynurenine downstream metabolites are bioactive and are translocated into the brain and are both neuroactive. If kynurenic acid is neuroprotective, quinolinic acid is however neurotoxic, thus suggesting that the balance between the two metabolites from the gut is influencing brain health and integrity ²⁴⁻²⁶. Moreover, arginine fermentation by gut bacteria leads to the formation of glutamate, and deamination of glutamate in that pathway leads to the production of the inhibitory neurotransmitter GABA ²². Microbial-derived GABA has been shown to have immunomodulatory properties since it regulates the proliferation of T cells. Similarly, the degradation of histidine by commensal bacteria leads to the formation of histamine, another important neurotransmitter involved in the central nociceptive response and motor control ²².

Secondary bile acids signaling pathway. Primary bile acids (BAs) are synthesized from cholesterol in the liver. They function as ‘detergents’ for the digestion of lipids and lipid-soluble vitamins in the intestines, facilitating their absorption by the GI tract. If the majority of primary

BAs are recycled in the liver, a small fraction is kept in the GI tract and are modified by colonic gut microbes. At the mechanistic level, intestinal microbes deconjugate primary BAs into secondary BAs, such as deoxycholic acid, that have antimicrobial properties, thus controlling the bacterial population density in the gut ²⁷. Importantly, some pathogenic bacteria can use secondary BAs to their advantage for virulence mechanisms ²⁸. BAs are integrated into signaling pathways regulating their own synthesis and transport but also for the regulation of lipid and glucose metabolism, as well as energy homeostasis in the host ²⁷, highlighting the importance of the secondary BA signaling pathway for host's metabolic health.

Short-chain fatty acids and host metabolic health. Gut microbes are essential for the energy extraction from non-digestible polysaccharides for the production of beneficial bioactive metabolites, the short-chain fatty acids (SCFAs). SCFAs include three main molecules: butyrate, propionate and acetate. These important microbial metabolites represent 6 to 10% of the host daily energy requirement ^{22,29,30} and are produced mainly by fiber-degrading species of the *Bacteroidetes* and *Firmicutes* phyla ³¹. For instance, butyrate is specifically produced by *Lachnospiraceae* bacteria while propionate is synthesized by *Bacteroides* and some *Clostridium* species ²⁷. As SCFAs derive mainly from the digestion of dietary fibers by the gut microbiota, the SCFAs pool depends directly on the proportion of fibers in host diet, and gut microbes are able to adapt to variations of fiber intake to produce the daily requirement of SCFAs for the host. For instance, Sonnenburg and colleagues ³² revealed that the SCFA-producing bacterium *Bacteroides thetaiotaomicron* modulates its metabolic activities depending on the source of fiber-based polysaccharides. A diet rich in fibers promotes the upregulation of genes from *B. thetaiotaomicron* involved in their catabolism. On the other hand, the absence of polysaccharides in the diet is detrimental for the growth of fiber-degrading microbes, such as *B. thetaiotaomicron*, and promotes the degradation of the mucus layer. Indeed, a low fiber-based polysaccharide diet favors a pool of genes implicated in the fermentation of host mucus glycans ³². Consequently, mucus layer reduction increases the risk of pathogen entry through the gut epithelium and the promotion of colitis in the host ³³. This important study indicates that some members of the gut microbiota are able to orientate their genome depending on nutrient availability and that metabolic capabilities of gut microbes are dynamic and plastic ^{22,32}. At the molecular level, the metabolic functions of SCFAs are mediated by the binding to the G-protein coupled receptors 43 and 41 (respectively, GPR43 and GPR41), which are expressed along the GI tract, as well as multiple other cell types such as immune cells, adipocytes and brain cells ³⁴. This widespread expression thus allows SCFAs to protect

the host from inflammation, well beyond the GI tract. In particular, suppression of GPR43 triggers gut inflammation in mice whereas activation of GPR43 by acetate protects against colitis^{34,35}. SCFAs are also involved in the regulation of hepatic lipogenesis, gluconeogenesis, the synthesis of cholesterol and insulin sensitivity. For instance, butyrate is the main source of energy for intestinal epithelial cells (IECs) and is involved in the intestinal barrier function by the promotion of tight junction proteins and mucin production by intestinal Goblet cells²². Propionate also serves as a substrate for IECs. Acetate, which is the most abundantly produced SCFA, is converted by the host into acetyl-coenzyme A, a crucial co-factor for many metabolic pathways³⁴, and is important for bacterial growth^{27,34}. In humans, populations consuming a low-fiber Western diet *vs.* African rural communities with a high-fiber diet have distinct gut microbiota composition, the latter population showing a more diverse microbiota and a decreased risk for metabolic diseases. Indeed, children from rural communities display an increase in the *Bacteroidetes* phyla, and thus an augmentation of fecal SCFAs levels, as well as decreased levels of potentially pathogenic bacteria (*i.e.*, *Escherichia*, *Shigella*). Moreover, SCFA-producing bacteria, especially from the *Bacteroidetes* phyla, protect from the establishment of metabolic diseases and contribute to the maintenance of a healthy body weight and controlled adiposity overall^{31,36,37}.

Diet-gut microbiota interactions thus have important implications for host physiology and metabolism. Of note, the gut microbiota contributes to the transformation of plant-derived polyphenols and the degradation of some xenobiotics^{7,22,38}. Altogether, these important studies underscore the importance of a balanced diet for a healthy gut microbiota. The gut microbiota adapts to host diet and fulfills its role of mutualistic microorganisms for dietary energy extraction and nutrition. Thanks to the sheer number of genes composing the gut microbiome, it participates in the synthesis of essential bioactive molecules that have consequences for host physiology and health in general³⁹.

1.2.2 Role of microbiota in the GI tract development and function

The intestinal epithelium is composed of different cell types, including intestinal epithelial cells (IECs), Goblet, Paneth and enteroendocrine cells (EECs). This complex cell layer is at the interface between the trillions of microbes inhabiting the gut lumen and the host, and communicate with it in a bidirectional manner (see **Figure 2**).

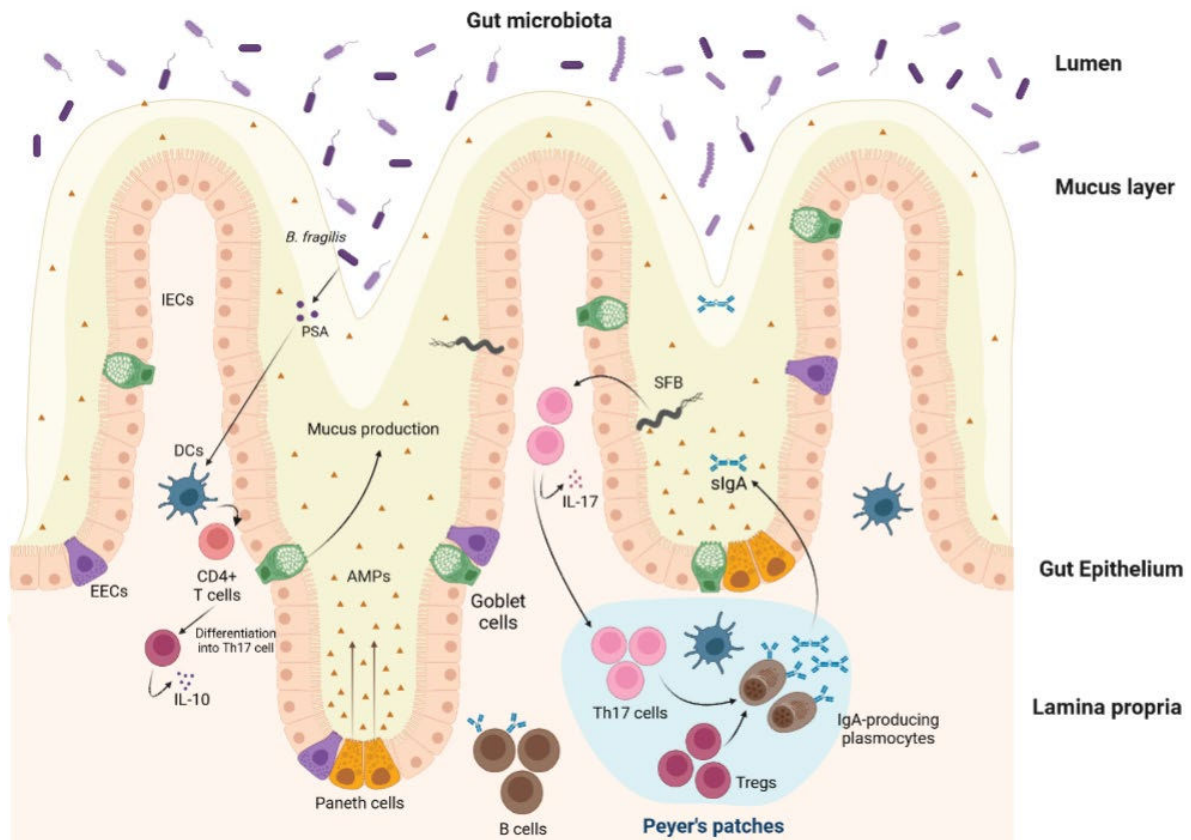


Figure 2. The interplay between the intestinal cell barrier, the mucosal immune system and the gut microbiota. The intestinal epithelium is a complex and well-organized microenvironment at the interface between the gut microbiota and the host, that includes IECs and specialized cells. First, Goblet cells produce mucus to prevent gut microbes to be in direct proximity with the IEC layer. At the intestinal crypts, Paneth and EECs produce antimicrobial peptides (AMPs) into the gut lumen. Underneath, in the *lamina propria*, Peyer's patches provide a cellular and adaptive protection against external microbial threats and includes IgA-producing plasma cells that secrete dimeric immunoglobulin A into the gut lumen. Secreted IgA (sIgA) is the most produced antimicrobial molecule at the mucosal layer, and can also be provided by activated B cells in the lamina propria. Helper T17 cells (Th17) and regulatory T cells (Tregs) populations are responsible for class switch of B cells secreting sIgA. Segmented filamentous bacteria (SFB) belong to the few bacteria that adhere to epithelial cells and promotes Th17 cells differentiation and production of interleukin-17 (IL-17) cytokine, that regulate the inflammatory status in the gut and constitutes a first line of defense against invading bacteria. Likewise, Th17 cells and Tregs produce IL-17 and IL-22 cytokines both in Peyer's patches and within the *lamina propria*. These cellular and humoral defense strategies allow the control of microbial populations at the intestinal barrier. Importantly, different populations of dendritic cells (DCs) are responsible for the sampling of microbial antigens, which promotes the development of Th17 cells and Treg cells. For instance, a bacterial polysaccharide A (PSA) derived from *B. fragilis* is sufficient to induce the differentiation of CD4+ T cells into IL-10-producing-Tregs, through antigen-presenting DCs and the Toll-like receptor 2

(TLR-2) signaling pathway. Figure adapted from *Muniz et al. (2012)* and *Zheng et al. (2020)* ^{40,41}. Created with BioRender.

Influence on intestinal barrier morphology. Studies in germ-free (GF) animals allowed a better understanding of the impact of the gut microbiota on GI structure and function. GF animals display several GI morphological impairments, the best known of which being a significant enlargement of the cecum, the most inhabited GI tract segment in conventionally-colonized rodents ⁴². In addition, GF animals display intestinal malabsorption, characterized by a reduction of the intestinal area, and impairments of villi morphology, among other defects ⁴³. Moreover, the gut microbiota reinforces the gut barrier integrity through its influence on epithelial cell desmosomes ⁴⁴ and tight junction protein complexes, (*i.e.*, occludin, claudin, *zonula-occludens*, ZO; and junction-adhesion molecules, JAMs) ⁴⁵⁻⁴⁷. Importantly, the absence of commensal microbes impacts directly intestinal cell turnover, tissue homeostasis and repair ^{48,49}.

Influence of microbiota on the development and function of the ENS. The ENS is plastic even during adulthood, since underdevelopment of the ENS in GF adult animals can be reversed with colonization with a conventional gut microbiota. Gut microbial colonization is paramount for the excitability of adult myenteric neurons, as GF mice show a slower and less reactive myenteric nervous response to an electric stimulation *ex vivo* ⁵⁰. Recently, *De Vadder and colleagues (2018)* showed that the gut microbiota signals through 5-HT₄ receptors to restore the maturation of myenteric neurons and fibers in adult GF rodents ⁵¹. Thus, these important findings highlight the importance of gut microbes-ENS interactions to establish and maintain the enteric neuronal networks.

1.2.3 Role of microbiota in the education and maintenance of the immune system

Production of antimicrobial molecules. Microbiota-host interactions at the gut mucosal barrier are indispensable for the development and maturation of the immune system. The absence of microorganisms leads to significant immunological defects in GF animals, such as a lack in several T cell populations that is observed until adulthood ⁴⁹. Several gut cell types, including IECs, are able to respond rapidly to microbial signals by the production of antimicrobial peptides (AMPs) (*e.g.*, lectins or defensins) and lysozymes. In the intestinal crypts, enterocytes and Paneth cells are activated by increased microbial numbers, which results in the secretion of these antimicrobial molecules ⁴⁶ (see **Figure 2**). Furthermore, IECs also produce cytokines to control commensal populations in the gut lumen ⁵². The most produced

immune molecule by the host in the mucosal milieu is the secreted immunoglobulin A (sIgA), which is also modulated by gut microbes⁵³ (see **Figure 2**). Indeed, GF mice have significantly lower levels of sIgA in their GI tract compared to conventionally-colonized animals⁴⁹. In a landmark study, *Suzuki et al.* (2004)⁵⁴ has shown that transgenic mice unable to produce sIgA display over-colonization of the small intestine with segmented filamentous bacteria (SFB). Interestingly, sIgA reconstitution in the intestinal *lamina propria* restores normal microbial numbers and decreases local immune system activation (*e.g.*, a decrease in B cell activation)⁵⁴, suggesting that host-produced sIgAs, but also antimicrobial molecules, are required for both the control of microbial density and the maintenance of a balanced mucosal immune system (see **Figure 2**).

Production of microbial-derived metabolites. Besides the recognition of microbial-derived components to modulate the host immune response, commensals also utilize their metabolites, such as SCFAs, to regulate the inflammatory state in the GI tract. In particular, SCFAs have anti-inflammatory properties notably by their capacity to stimulate antigen-presenting cells producing anti-inflammatory cytokines such as TGF- β , IL-10 and IL-18. For instance, acetate and propionate reduce the production of pro-inflammatory cytokines^{22,52}. AMP production is also induced through microbial-derived SCFAs, such as butyrate⁵⁵. Similarly, gut microbes also depend on the aryl hydrocarbon receptor (AhR) to regulate the development and function of the immune system, through the recognition of microbial metabolites or plant-derived phytochemicals. AhR allows the maturation of intestinal lymphoid cells in the gut, the formation of intestinal lymphoid follicles (ILFs) and the regulation of Treg cells pools and IL-22 production. It also induces the differentiation of Tregs through DCs by upregulating IDO and retinoic acid, a microbial metabolite derived from vitamin A⁵².

Influence on immune cell maturation in the gut. In the intestinal crypts, the gut microbiota promotes the development of secondary lymphoid structures, also called gut-associated lymphoid tissues (GALT)⁵⁶, as well as the development of mesenteric lymph nodes (MLNs). The GALT comprises both Peyer's and cecal patches, that are respectively located in the small intestine and in the cecum in rodents. GALT signaling is important in the microbiota-gut-immune axis as it enables the recognition of microbial commensals and pathogens within the GI tract. It also induces the maturation of ILFs in the intestinal *lamina propria*⁵⁷ (see **Figure 2**). Our colonizing microbes also affect the differentiation of Tregs and T helper cells (Th cells). Gut commensals induce innate lymphoid cells (ILCs) differentiation, such as IL-22-producing NKp46+ ROR γ t+ lymphoid cells⁵² and promotes gut barrier integrity⁵⁸ (see **Figure 2**). Some

studies even identified specific bacterial species involved in this immunomodulation, including *Lactobacilli*, *Bifidobacteria*, *Bacteroides* and *Clostridia*. For instance, an important study from *Mazmanian and colleagues* demonstrated that a polysaccharide derived from *B. fragilis* drives the general splenic maturation, the development of splenic CD4+ T cell populations and corrects Th1/Th2 cells imbalance in GF mice. Another report showed that *L. reuteri* also participates to Treg maturation and response, resulting in the production of the anti-inflammatory IL-10 cytokine ⁵⁹. In addition, SFB induce the activation and recruitment of IL-17 and IL-22-producing Th17 cells from the *lamina propria*, which facilitate the protection against pathogens, such as *Citrobacter rodentium* in the colon of GF mice ⁶⁰ (see **Figure 2**). The intestinal microbes are thus paramount for the establishment of a balanced inflammatory and immunological profile in the host, especially at the mucosal interface. Importantly, this specific zone of interface is at the origin of the pleiotropic effects of microbes on host health, including their influence on host's nervous systems.

1.3 The Microbiota-Gut-Brain axis

With the invention of the germ-free (GF) animal model in the 1950's-1960's, the understanding of the influence of our commensal microbes on health considerably improved ⁶¹. As discussed above, the GF animal model is structurally and functionally abnormal across numerous physiological systems, including the ENS and the CNS ⁶¹. Surprisingly, it has only been about the two past decades that our commensal microbes have been considered as a crucial third component in the gut-brain crosstalk, which led to the emerging concept of the 'Microbiota-Gut-Brain axis'. Important studies point out to potential signaling pathways linking the gut microbiota and the brain, which would underlie their complex, yet to be understood, interactions (see **Figure 3**).

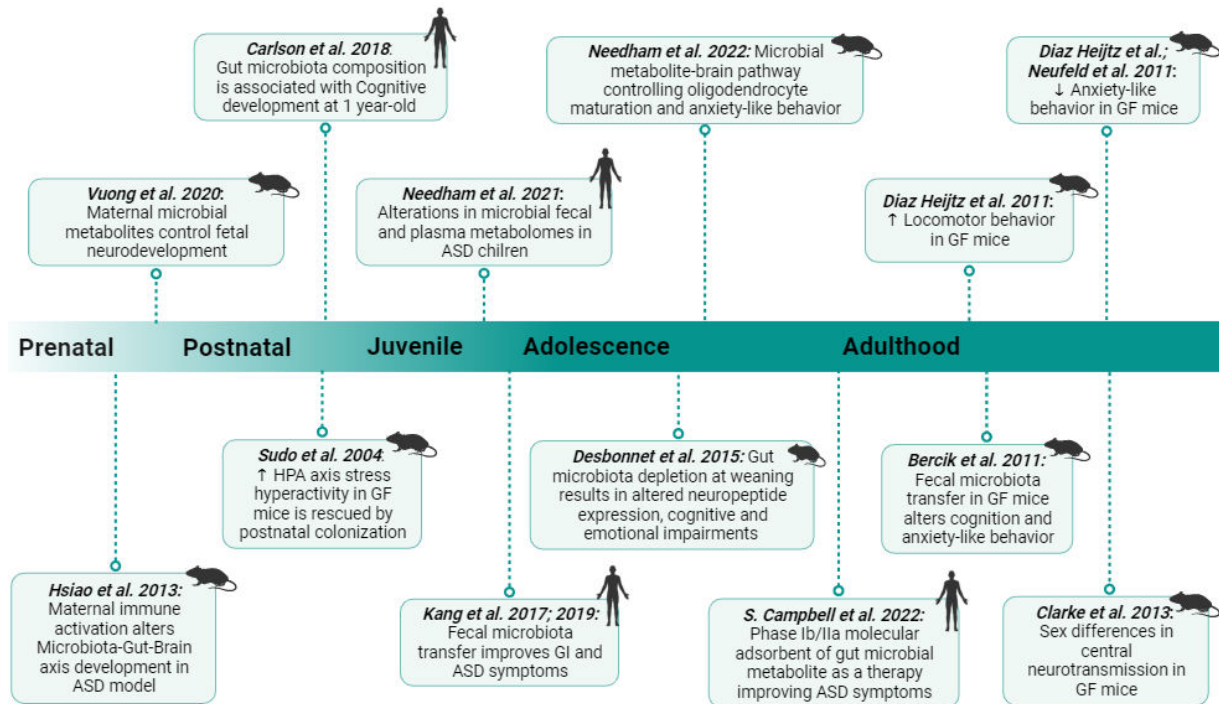


Figure 3. Pioneer studies on the development of the Microbiota-Gut-Brain axis and the relevance for neurodevelopmental disorders. This schematic timeline gives an overview of pivotal work in Microbiota-Gut-Brain axis research, and that provided important insights into the role of the gut microbiota in brain development, behavior, and by contrast, the influence of gut microbes on abnormal neurodevelopment such as in autism spectrum disorder (ASD). Other abbreviations: HPA: Hypothalamo-pituitary-adrenals axis, GF: Germ-free. Figure adapted from a presentation by Prof. Jane Foster (UT Southwestern, Dallas, Texas) at the *Mind Mood & Microbes pre-conference event (2023)*. Created with Biorender.

One of the first milestones to the Microbiota-Gut-Brain axis field was the findings of *Sudo and colleagues*⁶². They demonstrated for the first time a dysregulated HPA axis and an exaggerated central and peripheral responses to acute stress in GF mice⁶². Importantly, as neurodevelopmental disorders (NDD) are characterized with abnormal stress reactivity and altered anxiety levels, this pioneering work paved the way for the understanding of the impact of gut microbes on the central development of stress coping and the risk for stress-related disorders, such as NDD. Then, seminal studies^{63,64} described the behavioral phenotype of the GF mouse model and revealed that the absence of microbes leads to locomotor hyperactivity⁶³ and results in an anxiolytic state^{25,63,64}. Importantly, these behavioral impairments are accompanied with decreased levels of synaptic-related genes (*e.g.*, brain-derived neurotrophic factor, BDNF) in the hippocampus and the basolateral amygdala, two key brain regions

involved in stress-related behaviors ^{25,63,64}. Likewise, many neurotransmitter signaling pathways are dysregulated in the GF mouse, including catecholaminergic and serotonergic pathways ^{25,63,64}, especially in the hippocampus, which is a central hub for the regulation of cognition and socio-emotional behaviors. Others ⁶⁵ have confirmed that brain neurochemistry and anxiety-like behavioral traits are directly transferable to GF mice through fecal microbiota transplant (FMT), confirming that the gut microbiota controls the development of brain neurochemistry related to stress and anxiety in mice. Later, *G. Clarke and coworkers* ²⁵ demonstrated that the influence of microbial cues on the hippocampal serotonergic pathway functioning is sex-dependent and that microbial colonization of the GF gut at weaning fails to correct the neurochemical disturbances in these mice. This suggests that there are temporal windows during which gut microbes influence the proper development of brain neurochemistry. Indeed, *E. Hsiao et al.* ⁶⁶ demonstrated that the prenatal period is determinant for the development of a functional microbiota-gut-brain axis later in life. Disruption of the maternal gut microbiota following immune activation results in gut dysbiosis, GI barrier defects and socio-emotional abnormalities later in offspring, mirroring the physiological impairments observed in NDD. They also discovered that specific commensal bacteria of the gut microbiota, such as *B. fragilis*, modulate host metabolites. Importantly, this bacterial modulation rescues ASD-related impairments of the microbiota-gut-brain axis later in young adulthood ⁶⁶. Altogether, these important findings indicate that maternal gut microbes during fetal life impact the developmental trajectories of the MGB axis, very likely through their influence on host metabolome. Early adolescence is also another critical period during which the gut microbiota is important for brain development and socio-emotional behaviors. Antibiotic depletion from the weaning period until adulthood recapitulate the behavioral deficits observed in GF mice, including ones affecting cognition, anxiety-like and social behaviors, and are associated with alterations in the tryptophan and 5-HT pathways, synaptic-related signaling and socially-relevant neuropeptides, such as oxytocin (OXT) and vasopressin (AVP) ⁶⁷. Thus, this period would be important for the modulation of gut microbes on host neurotransmitter signaling pathways and neuropeptidergic pathways relevant to neurodevelopmental and neuropsychiatric disorders. Recently, Sarkis Mazmanian's laboratory ⁶⁸ identified differential metabolic profiles in a NDD mouse model, and that some microbial metabolites are able to cross the blood brain barrier (BBB) to directly interact with the brain. In particular, abnormal elevation of the metabolites 4-ethylphenyl sulfate (4-EPS) and 4-ethylphenol (4-EP) contribute to the reduced axonal myelination seen in this mouse model ⁶⁸, thus leading to abnormal neuronal connectivity and altered anxiety-like behavior, that might be at the origin of such disorders.

These pivotal pre-clinical findings were confirmed later, at least partly, in humans. Indeed, the gut microbiota composition in one-year-old infants is strongly correlated with cognitive development ⁶⁹, confirming that gut microbial colonization at this period may regulate brain development and behavior also in humans. Further, microbiota transfer in children with autism from healthy donors improves significantly their GI impairments and socio-emotional behavioral abnormalities ⁷⁰. This microbiota-based therapy was shown to be effective and safe in the long-term period, as ASD children that received a microbiota transplant show sustained improvements in GI function and emotional behaviors ⁷¹. Very recently, the team of Sarkis Mazmanian described significant differences in the metabolic profiles of ASD and neurotypical children, and these differences are mainly affecting oxidative stress, mitochondrial function as well as phenolic microbial metabolites, recapitulating their pre-clinical findings on gut microbiota-derived 4-EP and 4-EPS levels in mice ⁶⁸. Moreover, some ASD-related metabolite changes correlated with autism diagnosis and severity and was transmitted to mice via FMT ⁷², indicating that microbial metabolites might be involved in the onset and/or progression of autism. Promisingly, targeting ASD-related metabolites, such as 4-EPS or 4-EP, with the specific pharmacological adsorbent AB-2004 proved to be effective, as it ameliorates GI impairments and non-core behavioral symptoms of adolescents with ASD ⁷³. The phase Ib/IIa trial of this gut-restricted therapeutic molecule ⁷³ revealed to be safe for the treated patients. This work is a revolutionary advance in the treatment of ASD and confirmed that NDD, such as ASD, are largely dependent on the production of microbial metabolites. Nonetheless, other signaling pathways in the Microbiota-Gut-Brain axis might be at risk for the onset/progression of NDD.

1.4 Biological Signaling Pathways in the Microbiota–Gut–Brain Axis

These pioneering studies have paved the way to understand the potential signaling pathways along the microbiota-gut-brain axis, which are further described below (see **Figure 4**).

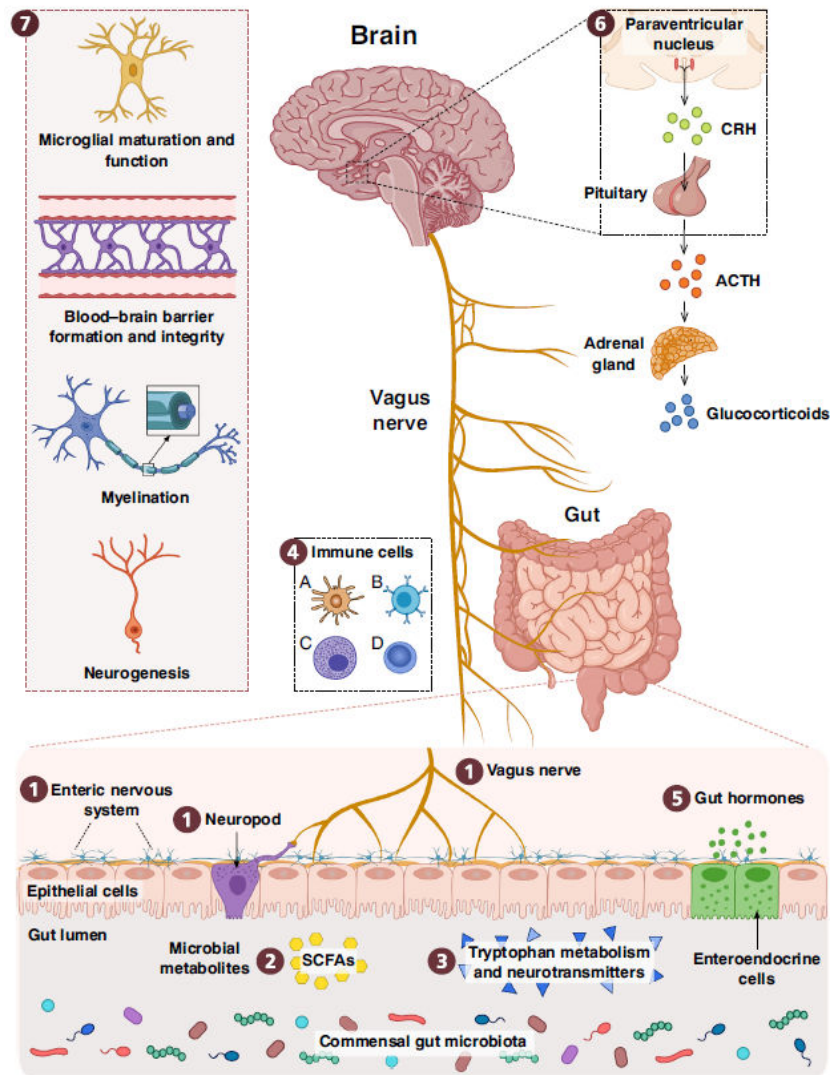


Figure 4. Potential signaling pathways of the Microbiota-Gut-Brain axis. The gut microbiota, the GI tract and the brain are communicating in a bidirectional manner, through several potential direct or indirect signaling pathways. A large body of research have helped to decipher the potential signaling pathways between these three components, and include (1) bidirectional gut-to-brain vagal afferents, which nerve endings are receiving signals from gut microbes via the autonomic nervous system (ANS) and the ENS, or through specific EECs, called neuropods; microbial-derived metabolites including (2) the short-chain fatty acids (SCFAs; butyrate, acetate and propionate); (3) Tryptophan metabolites and neurotransmitters (*e.g.*, 5-HT); (4) Release of cytokines by immune cells, including (A) dendritic cells, (B) B cells, (C) mast cells and (D) T cells and (5) gut hormones (*e.g.*, GLP-1 or PYY) produced by EECs. These molecules can affect brain activity and function either via vagal activation or reach the brain through the systemic circulation. In turn, these signals affect central pathways such as (6) the hypothalamic-pituitary-adrenals (HPA) axis, that is modulated by the vagus nerve through the nucleus tractus solitarius (NTS) projecting to the hypothalamus. Hypothalamic corticotropin-releasing hormone (CRH) neurons activate the pituitary gland to produce adrenocorticotrophic hormone (ACTH), which will

activate the adrenal gland to secrete glucocorticoids (e.g., corticosterone in rodents) modulating GI tract function. Gut commensals are shaping several neurodevelopmental trajectories in early life, potentially through these gut-to-brain signaling pathways, including (7) microglial maturation and function, BBB integrity, myelination and neurogenesis and neuronal migration, among other processes. Figure from *Gonzalez-Santana and Diaz Heijtz (2020)* ⁷⁴.

1.4.1 Neuronal pathways

The neuronal signaling pathway between the GI tract and the brain is supported by the ENS, and more specifically the autonomic branches of the ENS (i.e., the sympathetic and parasympathetic nerves). Thus, due to the dense and autonomous neuronal networks in the gut, the ENS is often referred to as ‘the second brain’. Although the ENS has the capacity to function autonomously from the CNS to control GI function, it uses the vagus nerve to communicate with the brain. The vagus nerve belongs to the parasympathetic branch of the autonomic nervous system (ANS) and represents the most direct route linking the GI tract and the brain, from the stomach to the transverse colon and rectum. Vagal afferents include axon terminals within the enteric smooth muscle layers, from enteric neurons in the intestinal mucosal cell layer ⁷⁵. As such, the gut is able to provide sensory information about its internal physiological state to the brain, and the brain sends back information via vagal motor efferents to the GI tract ⁷⁶. A large body of evidence have shown that vagal afferents also detect chemical signals from the gut and its microbiota, including cytokines, peptides, hormones and neurotransmitters ⁷⁷ (see **Figure 4**). For instance, neuropods, which are EECs from the IEC barrier, are innervated by direct vagal efferents and signals back to the vagus nerve through glutamate signaling ⁷⁵. Another recent study ⁷⁸ has demonstrated that optogenetic activation of vagal afferents of the upper gut promotes reward-related behavior in mice in a glutamate-dependent manner. Importantly, a growing number of studies revealed the impact of beneficial gut commensals on the vagal gut-to-brain communication. For example, *Lactobacillus rhamnosus JB-1* modulates the neurochemistry (i.e., GABA receptor gene expression) and anxiety- and depressive-like behaviors through the vagus nerve, activating vagal nervous endings within minutes after exposure to the probiotic strain ^{79,80}. Another lactobacillus strain, *L. reuteri*, acts on the vagus nerve to improve social behavioral deficits of several ASD mouse models ⁸¹, suggesting that gut microbes use the vagal gut-to-brain pathway, potentially through the glutamate and GABA signaling, to regulate the brain and associated behaviors. However, the exact underlying mechanisms remain to be elucidated.

1.4.2 Enteroendocrine signaling

Enteroendocrine cells (EECs) represent a restricted and specific population of cells of the gut epithelial layer that sense nutrient levels in the intestinal lumen. Their main function is the regulation of nutritional homeostasis and metabolism via the production and secretion of gut hormones such as the glucagon peptide-like 1 (GLP-1) and the peptide YY (PYY), two gut hormones that centrally modulate feeding behavior and satiety⁷⁷. The two best described types of EECs are the EEC L cells and the enterochromaffin cells (ECs)⁷⁷. Both EEC types can be stimulated by the gut microbiota to modulate brain function and feeding behavior (see **Figure 4**). Similarly, it was found that the gut microbiota is able to produce an antigen mimetic of the central appetite-regulating neuropeptide α -MSH, that activates the secretion of GLP-1 and PYY peptides from EEC L cells. This bacterial-derived protein, called caseinolytic protease B (*ClpB*), was shown to decrease food intake when delivered in the stomach of mice⁸² and is elevated in sera of patients with eating disorders⁸³. This raises the question about whether the gut microbiota can modulate host feeding behavior through a potential action of *ClpB* on the secretion of GLP-1 and PYY peptides. Interestingly, depletion of the gut microbiota protects Western diet-fed mice from hypothalamic inflammation and leptin resistance in a GLP-1R-dependent manner, suggesting that gut microbes, potentially through their modulation of GLP-1 release in the GI tract, acts on the central control of host nutritional homeostasis and metabolism⁸⁴. Importantly, ECs produce the majority of the 5-HT pool in the GI tract, a neurotransmitter that is essential for both the ENS and the CNS. There might be an indirect pathway by which the gut microbiota modulation of ECs would influence the brain, which is its ability to modulate tryptophan levels, the molecular precursor of 5-HT (see **Figure 4**). For instance, gut microbes are able to upregulate the first rate-limiting enzyme of 5-HT biosynthesis Tph1 in ECs²³. Altogether, this suggests that intestinal tryptophan levels, and indirectly central 5-HT levels, could depend on gut microbiota-ECs interactions and gut bacterial enzymatic machinery.

1.4.3 Neuroendocrine pathway – communication with the HPA axis

The HPA axis is the main neuroendocrine network that controls stress responsiveness in mammals and was the first described central system to be regulated by the gut microbiota from *Sudo and coworkers* in 2004⁶². This hypothalamic neuroendocrine network involves the production and secretion of the corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus, itself activating the secretion of the adrenocorticotrophic hormone (ACTH) from the anterior pituitary. In turn, ACTH reaches the

general circulation and stimulates the production of glucocorticoids from the adrenals. The HPA axis is a fine-tuned system involving a negative feedback loop with the action of peripheral glucocorticoids on glucocorticoid and mineralocorticoid receptors (respectively, GRs and MRs) in the anterior pituitary, the PVN, as well as in limbic brain regions such as the hippocampus and the prefrontal cortex (see **Figure 4**). In their landmark study, *Sudo and colleagues* demonstrated that postnatal microbial colonization in mice was shaping the development and function of the HPA axis ⁶². More particularly, they showed that a mild restraint stress in GF mice leads to an exaggerated stress response, first peripherally, as characterized by elevated levels of serum ACTH and corticosterone. Centrally, GF animals display decreased gene and protein expression levels of corticoid receptors (*i.e.*, GR and MR) and decreased brain-derived neurotrophic factor (BDNF) in the cortex and hippocampus, following acute restraint stress. Furthermore, colonization with the commensal gut bacterium *Bifidobacterium infantis*, or feces from conventionally-colonized mice (*i.e.*, specific-pathogen free mice or SPF mice), rescues the overactivation of the HPA axis stress response to the acute stress stimulus, and interestingly, only during early postnatal life but not at later developmental stages. Thus, this pivotal study provided the first evidence that gut microbes are shaping brain development and behavior during a specific time window of development ⁶². Others confirmed these findings ⁸⁵, notably in chronic stress mouse models ^{86,87}, or in a stress-prone rat model ⁸⁸. However, antibiotic-induced depletion in adult rats that were chronically-stressed, show the opposite outcomes on the HPA axis stress responsiveness, suggesting potentially that the time window during which the gut microbiota is depleted and the fact that antibiotics do not fully suppress commensal microorganisms, are important factors for the effect of gut microbes on the HPA axis function. Although the mechanisms underlying these effects are not fully elucidated, some studies using beneficial bacterial strains, such as *Lactobacillus rhamnosus* ⁸⁹ or *Enterococcus faecalis* ⁹⁰, showed that they contribute to the attenuation, and thus to the balance of the HPA axis responsiveness in stressed mice. Interestingly, the beneficial effects of *L. rhamnosus* on HPA axis reactivity might be partially mediated by the vagus nerve ⁸⁹.

1.4.4 Neuroimmune and immune pathways

As mentioned before, the gut microbiota and the immune system are constantly in dialogue to regulate each other's functions, including microbial population density and the maintenance of local immunity. This interplay is enabled by multiple humoral and cellular pathways, that could mediate the influence of the gut microbiota on brain function and behavior (see **Figure 4**).

The peripheral immune system. The peripheral immune system, which comprises myeloid cells (e.g., DCs, monocytes and macrophages, neutrophils, mast cells, among other cell populations) are producing immunomodulatory molecules, such as cytokines and chemokines, that can signal to the brain ⁹¹ (see **Figure 4**). Although the influence of the gut microbiota on brain development and behavior via the immune system remains largely unknown, several studies have shown that gut microbes can be implicated in the release of inflammatory molecules from immune cells in the context of brain disorders ⁷⁷. For instance, GF mice appear to be more resistant to experimentally-induced multiple sclerosis (MS) compared to control mice with a SPF gut microbiota, and colonization with SFB leads to increased levels of the pro-inflammatory cytokine IL-17, secreted by Th17 lymphocytes ^{91,92}, suggesting that commensal microbes would participate in the progression of inflammation, characteristic of neurodegenerative disorders such as MS. Another study ⁹³ has also made a link between stress, the gut microbiota and the adaptive immune system. In particular, mice stressed chronically following social defeat stress display increased levels of IL-10-positive Treg cells, accompanied by a shift in the gut microbiota and social behavioral deficits, which supports the concept of gut microbiota-adaptive immunity crosstalk for the regulation of CNS functioning ⁹³. Similarly, the probiotic mixture VSL#3 (containing *Bifidobacteria* and *Lactobacillus* species and a *Streptococcus* strain) restores the peripheral monocyte activation and activated microglial in a mouse model of liver inflammation. In turn, this led to decreased neuroinflammation and attenuated sickness behavior ⁹⁴, highlighting the interaction of commensal bacteria interact with peripheral pro-inflammatory monocytes that activate central inflammation and behavior. Altogether, these studies are confirming a link between the gut microbiota and neuroinflammation, potentially implicating the immune humoral and cellular routes in the regulation of brain function and behavior, both in steady-state and inflammatory conditions.

CNS-resident immune cells. Besides the periphery, the brain harbors resident macrophages, referred to as the microglia, that function as cellular sentinels against potential pathological threats reaching the CNS. Microglial cells are highly plastic and can drastically change their morphology depending on the activating molecular signal, and thus, orchestrate physiological neuroinflammatory processes to return to homeostasis ⁷⁷. Recently, microglia has been shown to be important cell mediators in the microbiota-immune-brain axis. Indeed, *Erny and colleagues (2015)* ⁹⁵ have demonstrated that the absence or the antibiotic-induced depletion of the gut microbiota during adulthood leads to an immature microglial phenotype and abnormal cell morphology, leading to a reduced immune response to bacterial or viral challenges in adult

animals *in vivo*. Interestingly, restoration of the gut microbiota complexity or supplementation with microbial-derived SCFAs are sufficient to restore most of the microglial defects observed in GF or antibiotic-depleted animals. Thus, this pivotal study⁹⁵ showed that gut microbes signal to the brain, possibly through SCFAs, for the maintenance and maturation of microglia throughout life. Other CNS-resident innate immune cells, such as resident Ly6C^{hi} monocytes, are involved in the regulation of brain function via, but not exclusively, gut microbial signals⁹⁶. In this study, the authors demonstrated that voluntary exercise or probiotic treatment increases Ly6C^{hi} monocytes in the brain and rescue hippocampal neurogenesis and associated cognitive deficits observed in antibiotic-depleted adult mice. Interestingly, transplant with this monocyte population, coupled with physical exercise and probiotic supplementation, rescued these deficits. This suggests that this specific brain immune cell population is a potential relay between microbial signals from the gut microbiota and physical exercise to promote adult neurogenesis and related cognitive behaviors⁹⁶. Taken together, the above studies indicate that a complex and diverse gut microbiota is required for the maturation and maintenance of brain innate immune cells and subsequent brain function in adulthood.

1.4.5 Microbial metabolites signaling

The SCFA signaling pathway. So far, the most studied microbial-derived metabolites for its implication on CNS function are the SCFAs. Microbial-derived SCFAs very likely act on CNS function through either direct or indirect routes across the microbiota-gut-brain axis, such as the ENS, the vagus nerve, the immune system, as well as the enteroendocrine signaling pathway^{77,97}. As part of the ‘direct’ gut-to-brain route, pre-clinical reports provided the evidence of SCFAs entry into the brain^{30,98,99}. For instance, it was shown that SCFAs are detected in the human cerebrospinal fluid (CSF)⁹⁷, suggesting their passage through the BBB. SCFAs might also act on the brain independently from central expression of its receptor GPR41⁹⁷. For instance, microbial-derived propionate activates intestinal gluconeogenesis via peripheral GRP41 stimulation in the vagal afferents and spinal nerves of the portal vein. Thus, microbial-derived propionate regulates central metabolism via a potential direct vagal activation³⁰. Likewise, activation of GRP41 and GPR43 expression in enteroendocrine L cells and the vagus nerve promotes the expression and secretion of gut hormones GLP-1 and PYY^{97,100}, thus suggesting that SCFAs influence central regulation of appetite and food intake via an indirect route. In a more direct manner, acetate reduces food intake centrally in mice, and influences the activity of hypothalamic appetite-regulating centers⁹⁹. More specifically, acetate is transported via the CSF to enter into the brain and then incorporates into the glutamatergic and GABAergic

metabolic cycles in the hypothalamus. In turn, this influences the central neurotransmission of appetite regulation, leading to decreased food intake in mice ⁹⁹. As propionate is the most concentrated SCFA in the brain compared to acetate and butyrate ^{77,97}, it has been extensively investigated for its effects on the human brain. A clinical study ¹⁰¹ has revealed that propionate regulates reward-based eating behavior by acting on the striatum, an important brain area involved in reward behavior, potentially through a direct effect on central appetite regulation.

Furthermore, SCFAs have been found to protect the healthy brain against neuroinflammation and is a key molecule for the maintenance of BBB integrity. Indeed, a pivotal study in GF mice ⁹⁸ has shown that butyrate is beneficial for BBB integrity, by increasing tight-junction protein gene expression in endothelial cells of the frontal cortex and hippocampus. Also, lipopolysaccharide (LPS)-induced inflammation *in vitro* is reduced with propionate pre-treatment and even protects LPS-induced disruption of the tight-junction integrity in a BBB cell model ¹⁰², thus confirming the protective role of SCFAs at the BBB. This protective role is also extended to microglia since microbial SCFAs exposure rescue microglial morphological and functional impairments observed in GF mice ⁹⁵. Similarly, butyrate alone triggers epigenetic modifications in microglia of various brain regions, notably via histone acetylation ¹⁰³. Taken together, these findings strongly indicate that gut microbiota-derived SCFAs dampen neuroinflammation and provides an anti-inflammatory protection to the healthy brain ^{98,104,105} via the modulation of CNS-resident brain immune cells and BBB integrity. Importantly, this anti-inflammatory effect attenuates the LPS-induced depressive-like behavior in a mouse model of chronic neuroinflammation ¹⁰³.

The tryptophan/indole signaling pathway. Besides SCFAs, gut bacteria are capable of converting tryptophan into indoles and metabolic byproducts thanks to a microbial-restricted tryptophanase ^{24,106}. As mentioned above, indoles and derivatives are bioactive metabolites involved in the intestinal function, but were recently shown to regulate brain function and behavior. In particular, acute and high doses of indole in rats, leading to the production of indole-oxidized derivatives, oxindole and isatin, are translocated from the gut to the brain and induce locomotor deficits in a dose-dependent manner ¹⁰⁷. On the other hand, chronic but moderate overproduction of indole by indole-metabolizing bacteria in gnotobiotic rats increase anxiety- and depressive-like behaviors, and intraperitoneal injection of indole itself activates the vagal pathway. This shows a possible mechanism by which indole-producing bacteria affect negative mood-related brain areas and behavior, via either a direct transport to the CNS or the stimulation of vagal afferents in the gut ¹⁰⁷. Gut microbiota-derived indole also promotes adult

mouse hippocampal neurogenesis and related cognitive functions¹⁰⁸ since mono-colonization with indole-producing *E. coli* or indole supplementation in GF mice rescues neurogenesis deficits *in vivo*. At the mechanistic level, indole activates neuronal proliferation, upregulates synaptic-related genes (*e.g.*, synaptophysin, PSD-95), as well as the maturation of neuronal processes in the dentate gyrus of GF mice, and these beneficial effects are abolished with the constitutive deletion of their receptor AhR, revealing a Indoles-AhR-dependent neurogenic effect in the healthy hippocampus¹⁰⁸. In humans, fecal indoles and derivatives are associated with anatomical and functional connectivity of brain regions belonging to the extended reward network (*i.e.*, the amygdala, the nucleus accumbens or NAc, the anterior insula and the brainstem), as well as with food eating behavior in healthy subjects¹⁰⁹. Although there are no causative conclusions in this study, these findings support the possible mechanism by which microbial-derived indole and metabolic by-products would modulate brain reward circuits to regulate food eating behavior in humans.

Overall, these recent findings suggest that microbial-derived tryptophan/indole byproducts are important signaling molecules of the microbiota-gut-brain axis and are involved in a wide range of central processes, including the regulation of reward-related behaviors, mood and cognition, but also in pathological context such as neuroinflammation.

2 The microbial colonization processes and the developing brain

2.1 Assembly and maturation of the infant gut microbiota

2.1.1 Microbial transmission, colonization, and succession

The *in utero* colonization hypothesis. During pregnancy, the maternal physiology undergoes significant shifts in hormonal, immune homeostasis and metabolism to promote the healthy development of the fetus. As the gut microbiota and the host are in constant interaction to regulate these systems^{110,111}, it is not surprising that the pregnancy state involves significant changes in the maternal gut microbiota during this period. Despite a large debate on a potential *in utero* microbial colonization, especially with studies on the amniotic fluid, meconium (*i.e.*, fetal fecal material), and placenta¹¹²⁻¹¹⁴, the initial seeding of the gut microbiota is widely considered to occur at birth¹¹⁵ and the evidence of a prenatal microbiome in the fetal organs, appendices and meconium requires further investigation¹¹². The microbial colonization of the GI tract derives mainly from the maternal vaginal and intestinal/perineal microbiomes, although other microbial niches (*e.g.*, skin or oral cavity) and the hospital environment also contribute to

the initial microbial seeding. It also depends on various factors such as the mode of delivery or the hospital environment during parturition.

The maternal gut microbiota before and during pregnancy. Many studies demonstrate that the periconceptual/prenatal period is a critical time window during which commensal gut microbes and/or their metabolites influence the proper development of the offspring¹¹⁶. As mentioned before, the human adult gut microbiota varies across life and its fitness may vary according to the socio-economic status, ethnicity, lifestyle, diet habits and health status^{116,117}. For example, the pre-pregnancy health status, especially in the case of overweight and obese mothers, is associated with changes in the maternal gut microbiota at birth. Although only limited long-term consequences for the infant gut microbiota are observed¹¹⁷, further investigation is needed to understand whether maternal dysbiosis resulting from a poor health status before conception would impact the initial microbial seeding and the overall metabolic development of the child^{116,117}. Throughout the course of pregnancy, the maternal gut microbiota composition and status is dynamically changing. In humans, the maternal gut microbiota diversity undergoes a significant shift between the first trimester and the third trimester of pregnancy, which is characterized with a higher abundance of *Proteobacteria* (*i.e.*, bacteria that are commonly associated with inflammation, including *E. coli*) and a lower abundance of butyrate-producing bacteria, such as *Faecalibacteria*¹¹⁸. This shift also includes increased abundance of *Enterobacteria*, *Streptococci* and *Staphylococci*, that are facultative anaerobes transiently inhabiting the newborn's gut microbiota and prepare to the colonization by strict anaerobes^{110,118}. Importantly, the composition of the first-month neonate closely resembles to the maternal gut microbiota in the last trimester¹¹⁸. By comparison, women's gut microbiota in the 1st trimester display a higher abundance of *Firmicutes* (mainly from the *Clostridiales* family) over *Bacteroidetes*, similarly to non-pregnant women's gut microbiota. These findings indicate that the maternal gut microbiota is the main source of initial microbial colonizers and is thus paramount for the infant's gut microbiota fitness in early life. In addition, the authors of the study¹¹⁸ investigated the functional consequences of these temporal shifts in the maternal gut microbiota during pregnancy. Following FMT from women at the first and third trimester into GF mice, they have shown that GF recipients of third trimester mothers show a greater adiposity, hyperglycemia and lower insulin sensitivity, compared to mice transplanted with the gut microbiota of women in the first trimester¹¹⁸. These important results indicate a drastic gut microbiota remodeling in pregnant mothers, which is necessary for the

increased energy demand from the growing fetus in the last trimester of pregnancy and for the event of birth.

The maternal vaginal and oral microbiota. Other maternal microbial niches in prenatal life impact the initial microbiota colonization process ¹¹⁶. Similar to the intestinal microbial niche, the human vaginal microbiota undergoes a significant decrease in diversity over the course of pregnancy ¹¹⁹, and the early postpartum period ¹²⁰, with a progressive dominance of the *Lactobacillus* genus, including an increase in the relative abundance of several *Lactobacilli* species/strains (e.g., *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. vaginalis*, *L. iners*) whereas a decrease in anaerobic bacterial families, such as *Atopobium*, *Prevotella* or *Sneathia* is observed. Moreover, healthy pregnant women delivering at term, show a high stability in the vaginal microbiota compared to non-pregnant women ¹¹⁹. Interestingly, *Lactobacilli* are indispensable for the maternal microbiota equilibrium since many lactic acid bacteria (from the *Lactobacillus* genus) increase the vaginal environmental pH and produce antimicrobial molecules, thus preventing the entry of harmful microbes in the womb and a risk of infection-induced pre-term delivery ^{110,116}. Of note, differential microbial community structures between women delivering at term and women delivering pre-term (e.g., increase in *Prevotella* species, *Sneathia amnii* and decrease of *L. crispatus*) warrants further attention for the potential risk of pre-term microbial shift on the infant's initial microbial seeding and subsequent health outcomes. However, more evidence is needed to determine a vaginal microbial signature for the risk of pre-term delivery.

The beginning of colonization at birth. In a natural vaginal birth setting, the first microbial colonizers are facultative anaerobes (e.g., *Staphylococci*, *Enterobacteria*), which facilitates the transient gut colonization with strict anaerobes, such as *Bacteroides* and *Bifidobacteria* (belonging to the *Bacteroidetes* phylum) and *Clostridia* (e.g., from the γ -*Proteobacteria* phylum), and *Bifidobacteria* are predominant in the infant gut microbiota in the first months of life ^{116,121–125} (see **Figure 5**). With the precision of genome sequencing techniques in the last decade, the infant gut microbiota assembly and composition has been characterized at the species and strain levels. For instance, *Yassour and coworkers* ¹²⁴ demonstrated that the vertical transmission from mother to infant occurs in a two-fold process at birth. First, there is a systematic transmission with maternal dominant strains, including *Bifidobacterium longum*, *Bacteroides vulgatus*, *Bacteroides dorei*, *Bacteroides uniformis* and *Bifidobacterium adolescentis*, and occasional transmission with maternal secondary strains. The authors observed that maternal secondary strains are selected in the infant gut microbiota when they have a functional relevance for the infant. For instance, some *B.dorei* and *B.uniformis* strains

expressing genes for starch breakdown are more often selected in the infant gut microbiota because it will likely be useful for the digestion of polysaccharides from breastmilk ¹²⁴. Thus, this study reveals that vertical transmission of the gut microbiota in early life infancy does not occur at random, and maternal gut microbes are selected in the best functional interest for the healthy development of the baby. Vertical mother-to-infant microbiota transfer also occurs through breastmilk, itself colonized with various microbial niches such as the skin and the oral cavity ^{110,111,116}. In mice, oral exposure to *Lactobacilli* strains during late pregnancy, thus ending in the maternal GI tract, can be transferred to the newborn through the mammary gland and milk during lactation. Besides natural prebiotics (e.g., human milk oligosaccharides; HMOs), vitamins and maternal antibodies, the microbial composition of breastmilk evolves from birth until the end of lactation, and parallels important developmental milestones of the infant ¹¹⁰. Regardless of ethnic, geographical or delivery mode factors, it appears that the core breastmilk microbiota is enriched in *Weissella*, *Leuconostoc*, *Pseudomonas*, *Staphylococcus* and *Streptococcus* genera, with dynamic variations throughout the whole lactation period ¹¹⁰. Overall, these findings highlight the importance of breastfeeding (vs. formula feeding) for the establishment of a stable and complex gut microbiota in early infancy ^{111,126}.

Stabilization and maturation up to 2-3 years of age. At the transition between lactation and weaning, a stabilization and a complexification of the gut microbiota occurs with the dominance of the *Firmicutes* phylum, including *Lactobacilli* species ^{122,125} and leads to a relatively stable *Bacteroidetes/Firmicutes* ratio in the gut. At one year of age, the human gut microbiota progressively resembles to the maternal gut microbiota postpartum ¹¹⁸. There are an increase in the microbial α -diversity (e.g., elevated diversity within the infant's microbiome) and a reduced β -diversity (e.g., the gut microbiota composition of the infant progressively resembles to other individuals, such as other family members) ^{123,127}. However, some compositional and functional dissimilarities remain at one year of age, suggesting that the infant gut microbiota still needs to mature at this time of life ¹²⁷. It is now established that the gut microbiota acquires an adult-like composition and diversity around 2 to 3 years of age in humans ^{15,49}, and in the two weeks post-weaning in mice ¹²⁸. At the functional level, the dynamic shift of the gut microbiota during early infancy corresponds to a progressive maturation of the gut microbiota metabolic activity and functionality, with the colonization of commensals involved in the biosynthesis of amino acids (e.g., tryptophan-metabolizing bacteria) and vitamins, mainly belonging to the *Firmicutes* phylum ¹²⁷. For instance, *Akkermansia* and *Faecalibacterium* species appear in the infant gut microbiota during this period of life. These bacteria are important for the degradation of fibers

and produce SCFAs, and their appearance in the infant gut microbiota corresponds to the introduction of solid foods ^{110,116}.

2.1.2 Implications for the developing GI tract, immune and metabolic systems

Implications for the developing immune system. Microbial exposure during the perinatal period determines the development and maturation of the infant’s immune system and GI tract, and these effects might be long-lasting. The mucosal immune system is located in the intestinal epithelium, in the underlying *lamina propria* and in the submucosal lymphoid structure underneath, including Peyer’s patches, ILFs and MLNs. Some hematopoietic cells of the innate immune system are also ‘patrolling’ within the mucosa and include mononuclear cells, mast cells, macrophages, DCs and ILCs, that sense microbial antigens through the pattern recognition receptors (PRR) signaling pathway. Importantly, the intestinal expression of Toll-like receptors (TLR) develops postnatally in an age-dependent manner, that allows a progressive immune priming by the microbial colonizers ¹²⁹.

There is a sequential establishment of the immune system and the GI tract, paralleling the progressive microbial colonization of the infant gut (see **Figure 5**), that starts during pregnancy, followed by the birth event, lactation and finally the first years of life post-weaning.

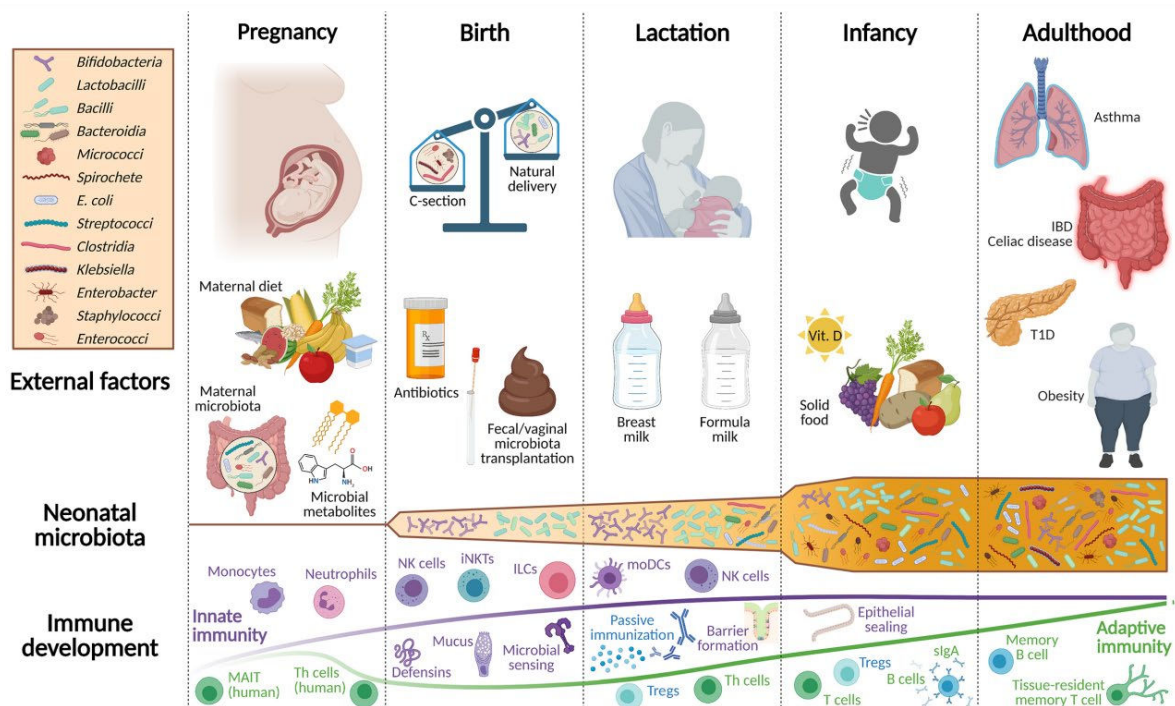


Figure 5. Parallel development of the microbiota, the mucosal immune system and the GI tract during the perinatal period, and factors influencing the developing gut microbiota. Microbial-derived components or metabolites are transferred from the mother to the developing fetus during the

prenatal period, and contribute to the development of innate immune cells, including monocytes and neutrophils. Of note, adaptive immune cells (e.g., MAIT cells, Th cells) were shown to be primed only in the human fetus. At birth, the first microbial colonizers (mostly *Lactobacilli* and *Bifidobacteria*) induce the maturation of a first-line of defense, including the development of NK cells, iNKT cells and ILCs in the gut. Paneth and goblet differentiate progressively from birth onwards. This process is enabled through innate immune receptors, such as PRRs, that allow microbial antigen sensing in IECs and DCs. During lactation, adaptive immune cells start to develop and include Tregs and Th cells (in mice specifically), and antigen-presenting cells such dendritic cells and NK cells function is primed in the intestinal compartment in mice. Furthermore, the maternal milk provides passive immunity through the transfer of immunoglobulins, microbial components and HMOs, that serve as prebiotics for the developing gut microbiota. As such, HMOs contribute to the diversification and increased abundance of the infant gut microbiota. During this critical postnatal period, colonizing gut microbes, including *Lactobacilli* and *Bifidobacteria*, promote the IEC barrier formation. At weaning, the infant's diet transitions from milk to solid foods, further increasing the abundance and diversity of the gut microbiota. This leads to the strengthening and 'final' maturation of the IEC barrier. Also, the 'memory' immunity of the adaptive immune system, supported by B and T cells, is initiated through gut microbial signals, and further increases the available immunoglobulin pools in the intestinal mucosa, such as sIgA. At 2 to 3 years of age, the infant develops an adult-like gut microbiota that enables the maturation of memory B cell pools and tissue-resident memory T cells. Depending on the developmental time window of this period, several external or internal factors influence the gut microbiota development and subsequently, the developing GI tract and immune system. During pregnancy, maternal diet influences the maternal gut microbiota and thus the maternal metabolites transferred to the fetus. At birth, the mode of delivery (c-section vs. vaginal delivery), the use of antibiotics and/or fecal and vaginal microbiota transplantation contribute to the initial seeding of the gut microbiota. During lactation, the quality of the milk fed to the infant will condition the shaping of the gut microbiota during lactation, and after weaning, solid foods and other types of factors contribute to the developing gut microbiota. In turn, these factors might contribute to the onset and/or progression of auto-immune and inflammatory diseases such as asthma, inflammatory bowel disease/ceeliac disease, Type I diabetes or obesity in adulthood. IBD, inflammatory bowel disease; sIgA, secretory immunoglobulin A; ILCs, innate lymphoid cells; iNKTs, invariant natural killer T cells; MAIT, mucosal-associated invariant T cells; moDCs, monocyte-derived dendritic cells; NK, natural killer cells; T1D Type I diabetes Th cells, T helper cells; Treg, regulatory T cells. Figure from *Kalbermatter et al. (2021)* ¹²⁹.

In the prenatal period, microbial-derived components or metabolites from the mother can be transmitted to the fetus through the placenta, subsequently preparing the immune system for later postnatal life ^{130,131}. In a pioneer study, *Gomez de Agüero and colleagues* ¹³² have shown

that transient colonization of GF dams during pregnancy impacts the mucosal innate immune system of the offspring and indicate that maternal microbiota provides a first-line immune defense to adapt to the rapid and dense postnatal colonization. First, there is a placental transfer of IgG, bound to microbial metabolites such as indole-3-carbinol, that partially mediates the recruitment of group 3 innate lymphoid cells (ILC3) in the *lamina propria* of 14-day-old pups¹³². Secondly, maternal colonization during gestation upregulates gene networks involved in mononuclear cell recruitment, Ig receptors and AMPs. In a context of maternal infection, maternal-derived IL-6 is also transferred to the fetus, and modifies epigenetically fetal intestinal epithelial stem cells. In turn, pro-inflammatory Th17 lymphocyte reactivity is enhanced at the intestinal barrier and confers a long-lasting protection against infection¹³³. However, these responses increase the susceptibility of gut inflammation in the adult offspring^{131,133}.

As microbes rapidly and densely colonize the infant gut at birth, the immature immune system must tolerate gut commensals while maintaining protection against potential pathogens¹³¹. GF studies have revealed that microbial colonization primes the activation of ILFs in the second postnatal week in mice⁵⁷, Peyer's patches¹³⁴ of the intestinal *lamina propria* and starts recruiting intra-epithelial lymphocytes, that have a role of immune surveillance in intestinal villi¹³⁵. Likewise, microbial colonizers triggers the recruitment of mature bone marrow-derived macrophages¹³⁶, promote the generation of ROR γ t⁺ regulatory T lymphocytes (Tregs)^{137,138} and the development of the intestinal lymphatic system circulation, allowed by MLNs¹³⁴. Similarly, it was established that tolerance of iNKT lymphocytes depends on exposure to gut colonizing microbes shortly after birth. Indeed, gut microbes in the newborn modulate chemokine CXCL16 expression epigenetically in iNKT cells, inducing the recruitment of these cells in the intestinal mucosa^{52,139}.

During the pre-weaning and lactation period, the offspring adaptive immune system starts to develop and is modulated by microbial colonization (see **Figure 5**)^{129,131}. At this period of life, microbial colonization shapes B lymphocytes and immunoglobulin repertoires in the intestinal *lamina propria*¹⁴⁰, and microbial-derived vitamin B2 metabolites are required for the development of mucosal-associated invariant T (MAIT) cells, involved in mucosal homeostasis and tissue repair¹⁴¹. During lactation, the breastmilk microbiota and microbial-derived metabolites also promotes the postnatal colonization of the infant and thus contributes to the education and maturation of the immune system¹²⁹. Breastmilk is a crucial vector of microbes (*e.g.*, *Bifidobacteria*¹⁴²), microbial-promoting components (*e.g.*, HMOs¹²⁹) and immunoglobulins that promotes a tolerogenic immune response¹⁴² towards the infant gut

microbiota postnatally, while providing a passive protection against potential pathogens. For instance, maternal breastmilk sIgA allows the compartmentalization of *B. fragilis* strains by anchoring them to the mucus layer, which enables a selective competition with other exogenous microbes in the gut lumen ¹⁴.

With the cessation of breastfeeding post-weaning, the gut microbiota composition diversifies, that triggers a strong immune response at weaning, that was termed recently as ‘the weaning reaction’ ^{130,138}. This transient immune response is characterized by high intestinal levels of pro-inflammatory cytokines (*e.g.*, IFN γ , TNF α), mediated by an increased generation of Tregs, until the fourth postnatal week in mice. Further, microbial SCFAs and host-derived retinoic acid activate Tregs, and authors have shown that this immune phenomenon at weaning is critical for the susceptibility to inflammatory diseases (*e.g.*, colitis, IBD) later in life ^{131,138} or food allergy ¹⁴³. Thus, these findings indicate that there is a specific time period for microbial antigen-immune interactions in the infant gut, that will result in a lifelong tolerance towards gut commensals and develop a functional immune system ^{131,144}.

Implications for the developing GI tract. Early microbial signals are required for the development of different intestinal cell types during the whole perinatal period. As stated before, the function of various intestinal cell types is already programmed in fetal life, notably with the exposure to metabolites derived from the maternal microbiota ¹³². Colonization of GF dams leads to upregulated transcription of genes involved in the differentiation of Paneth cells, goblet cells and precursors of IECs. Likewise, microbial antigens from the mother upregulate gene networks involved in the metabolism of xenobiotics, BAs, lipids and sugars ¹³², providing the infant with a ‘minimal’ enzymatic arsenal to face the massive exposure to microbial and exogenous antigens at birth. In the postnatal period, the intestinal epithelial barrier matures predominantly between the 2nd and 3rd weeks in mice, and its establishment is dependent on the gut microbiota. Indeed, gut colonizing microbes, such as *Lactobacilli* (*i.e.*, *Lactobacillus rhamnosus* GG) or *Bifidobacteria* strains (*i.e.*, *Bifidobacterium breve* strain UCC2003) promote the expression of tight junction proteins (*e.g.*, Claudin-3) ¹⁴⁵ and integrins ¹⁴⁶, that govern the gut epithelial barrier integrity. Likewise, intestinal mucins genes are upregulated following the neonatal supplementation with *B. breve* strain UCC2003, revealing that the probiotic strain would also be beneficial for the establishment of the physical mucus barrier in the intestines ¹⁴⁶. This strain also influences the development of intestinal epithelial stem cells in the small intestine, with the upregulation of genes involved in stem cell proliferation gut epithelial maturation ^{146,147}. This suggests that the *Bifidobacteria* dominance in the neonatal gut

microbiota has a critical functional importance for the development of the host GI tract. Mechanistically, gut microbial signals regulate the development of the intestinal epithelial cell barrier through the Myd88 protein, downstream of the PRR signaling pathway of the innate immune system, suggesting that the maturation of the intestinal barrier integrity by the gut microbiota in early life is dependent on the innate immune system¹⁴⁵, and more specifically through the TLR2-MyD88 signaling pathway^{148,149} that recognizes bacterial surface bioactive molecules. Moreover, it was shown that a soluble transcriptional factor, called the erythroid differentiation regulator-1 (Erdr1) is an important factor for intestinal cell proliferation and epithelial barrier structure in the GI tract¹⁴⁷. Erdr1 acts on intestinal cell proliferation through epigenetic modifications by the gut microbiota in the first weeks of life¹⁴⁷, and other reports show that DNA methylation of genes involved in the glycosylation of intestinal maturation is regulated by gut microbes during this specific period¹⁵⁰. Overall, these studies indicate that colonizing gut microbes in the first postnatal weeks promote the establishment and maintenance of the intestinal cell barrier, very likely through epigenetic modifications of genes involved in cell proliferation and renewal. At the weaning transition, corresponding to a dietary shift from breastmilk to solid foods, microbial-derived metabolites such as the SCFA butyrate, contribute to the gut barrier strengthening and maturation, that is reflected by an increased electrical resistance and a decreased permeability of the cecal tissue¹⁵¹. Correspondingly, the gut microbiota composition shifts rapidly at this transitional period, with a higher α -diversity and relative abundance in 25-day old rabbits pups. As the gut microbiota diversifies, microbial cecal SCFAs levels increase significantly¹⁵¹, and contribute directly to the maintenance of the developing intestinal barrier.

Similarly, the developing gut microbiota is required for the development of the ENS¹⁵². *Collins and colleagues*¹⁵³ have shown that GF mice at postnatal day (P) 3 develop an immature myenteric plexus in the jejunum and ileum¹⁵³. The minimal nerve and neuronal cell density observed in GF neonates was accompanied by an impaired inhibitory nitric oxide (NO) neurotransmission in the myenteric plexus, resulting in decreased gut motility in these intestinal segments. Antibiotic-induced microbiota depletion post-weaning confirm these results¹⁴⁹, since antibiotic-depleted juvenile mice display severe defects in GI neuromuscular and metabolic functions associated with impairments in intestinal tissue morphology, altered neurotransmitter signaling pathways (*i.e.*, NO, cholinergic and tachykinergic neurotransmission) and impaired neuronal and glial cell networks¹⁴⁹. This suggests that the gut microbiota colonization is involved in the development of enteric neurons regulating neurotransmitter signaling pathways

in the ENS and that mucosal glial cells also support the intestinal epithelial barrier integrity and renewal. Furthermore, molecular cues from the gut microbiota control the development of enteric glial cells, that migrate from the intestinal ganglionic plexi to the mucosal wall between the 1st and the 4th postnatal weeks in mice. This microbiota-host molecular interaction maintains enteric glial cell renewal and homeostasis in the intestinal *lamina propria* throughout life ¹⁵⁴.

Altogether, these studies indicate that the development and function of the GI tract and the mucosal immune system depend on commensal gut microbes and their metabolites, across the whole perinatal period. Essentially, the microbial-mucosal immune system cross-talk is required to maintain a balanced immune protection in the gut, and facilitates the essential metabolic and immune gut functions (see **Figure 6**).

2.2 Factors influencing the infant microbiota and health

Various factors influence the microbial colonization process in early life including the birth delivery mode, infant feeding mode (*e.g.*, breastfeeding vs. formula-based diet), and antibiotic exposure, among others (see **Figure 6 below**).

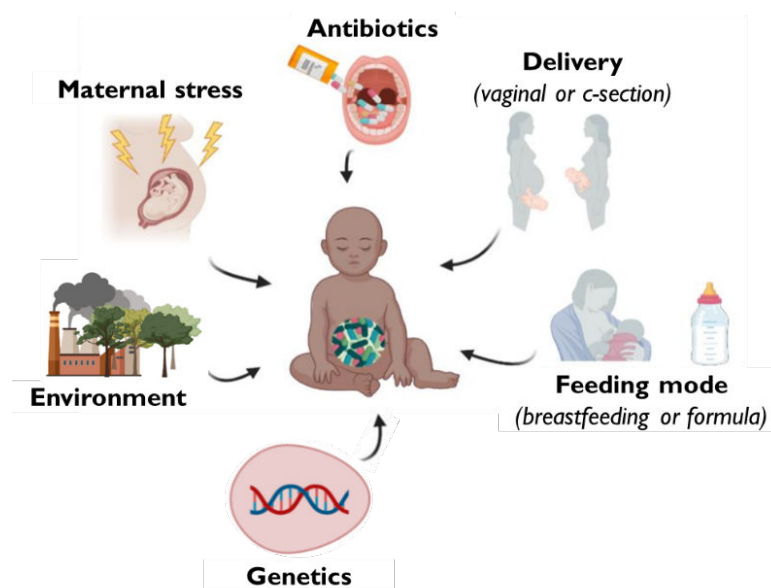


Figure 6. Factors influencing the gut microbiota colonization process in the infant. Several perinatal insults can interfere with a ‘normal’ microbial seeding and/or colonization in infants, including the mode of delivery (vaginal birth vs. c-section delivery, the latter reducing microbial seeding with vaginal and intestinal microbes at birth), neonatal feeding mode (natural breastfeeding vs. formula-based feeding), genetic background, environmental factors (exposure to pollution, pets, etc.), maternal stress, as well as perinatal antibiotic exposure, being the most encountered confounding factor with a healthy microbial colonization in the first years of life. Other factors such as maternal and/or neonatal infections,

gestational age or even exposure to other types of medication can influence the shaping of the early gut microbiota. Altogether, this suggests that overlapping of these factors, either from environmental or genetic origin, determine the establishment of the infant core microbiota. Figure adapted from Diaz Heijtz, (2016) and Borre et al., 2014^{155,156}.

2.2.1 Mode of delivery

The primary aspect for the assembly of the gut microbiota is the birth delivery mode, that was shown to be the main factor influencing the neonatal microbial composition^{157,158}. It is now widely established that the gut microbiota from vaginally-delivered children is different in composition and abundance compared to infants born from cesarean section (c-section) during infancy^{116,157–159}. Indeed, c-section-born babies present a larger population of skin (e.g., *Staphylococcus*, *Streptococcus*, *Corynebacteria*, *Propionibacteria*) and environmental (e.g., *Klebsiella*, *Clostridium*) microbes in their initial gut microbiota, whereas microbial communities of vaginally-delivered neonates are more diverse and mostly colonized with vaginal (e.g., *Lactobacillus*, *Prevotella*) and perineal/fecal bacteria (e.g., *Bacteroides*, *Bifidobacteria*, *Parabacteroides*, *Escherichia*) in humans¹⁵⁸. Thus, c-section-born infants show a delay in the colonization by *Bifidobacteria*, *Bacteroides* and *Enterobacteria*^{127,160,161}. In addition, they display a higher abundance of microbes that can be detrimental for health if in larger abundance, such as *Clostridium difficile*, that can cause serious GI infections^{115,158}. Moreover, vaginally-born and c-section-born offspring gut microbiomes still cluster differently in 8-week-old mice, suggesting a sustained effect of birth delivery mode in rodents' developing gut microbiome¹⁶². To compensate this discrepancy, Dominguez-Bello and colleagues¹⁶⁰ proposed that exposure to fresh vaginal secretions from the mother may restore partially the delay in microbial seeding in c-section-born infants. In addition, FMT from the mother to the c-section-born neonate led to a similar microbiota composition to vaginally-born infants up until three months of life¹⁶³, suggesting that maternal vaginal and fecal commensal microorganisms are paramount for a healthy microbial seeding of the infant GI tract, but transiently colonize the GI tract in humans compared to mice. Indeed, several studies have shown that non-intestinal originating microbes transiently colonize the infant gut, that result in a 'microbial convergence' with age between the gut microbiomes of vaginally-born and c-section infants^{116,158,161}.

The development of the immune system is also perturbed following c-section delivery. C-section delivery in mice results in a decreased tolerogenic response from the neonatal immune

system¹⁵⁷: T regs numbers are decreased in the MLNs and spleens of 5-week-old offspring mice, and DCs and anti-inflammatory cytokine IL-10 levels are dampened following c-section delivery¹⁵⁷, whereas invariant iNKT cells molecular markers (e.g., IL-4 and IL-15) are upregulated in the spleen¹⁶². Then, this could have consequences for the onset and development of inflammatory and/or auto-immune diseases later in life. Indeed, gut microbial differences between vaginal and c-section delivery modes at one-year-old influence the risk of developing asthma before 6 years of age, although other confounding factors may contribute¹⁶¹.

Importantly, consequences of birth delivery mode were reported for the development of the microbiota-gut-brain axis. During the postnatal period, c-section delivery affects the dendritic arborization process¹⁶⁴ and the development of neuronal networks in brain areas regulating social behavior, anxiety and cognition¹⁶⁵⁻¹⁶⁷. The effect of birth delivery mode on brain development and behavior might be related, at least partially, to the delay in the microbial colonization by *Bifidobacteria* during the neonatal period¹⁶⁶. Indeed, *Bifidobacterium breve* or *B. breve*-targeted prebiotic (i.e., a mixture of fructo-oligosaccharides and galacto-oligosaccharides) corrects the deficits in *Bifidobacteria* levels in the gut microbiota of offspring during early life, and partially restores social cognition, maternal attachment and early life social communication impairments. Clinically, c-section delivery has been moderately associated with poor school performances in adolescents¹⁶⁸ and minor risk of developing psychosis later in adulthood, although this may be confounded with genetic inheritance and other environmental and family factors¹⁶⁹. In another cohort of healthy male students¹⁷⁰, c-section delivery may have lasting negative consequences on psychological, exam-related stress and anxiety, compared to vaginally-born counterparts¹⁷⁰. These elevated stress levels are accompanied with increased plasma levels in IL-1 β and IL-10, revealing an increased inflammatory response to psychological stress and potential influence of birth delivery on the adult immune system functioning. Of note, the gut microbiota composition of young adult participants is similar between vaginally-delivered and c-section-born groups, indicating that (1) if the gut microbiota composition in humans is influenced by the birth delivery mode in early life, there is a microbial convergence later in life and (2) that the potential influence of gut microbes on lifelong brain performances and behavior would rather occur in infancy¹⁷⁰. Interestingly, *Castillo-Ruiz and colleagues*¹⁶⁷ have recently shown that the birth event itself, regardless of the delivery mode, triggers an inflammatory immune response in the newborn's periphery and brain. This inflammatory response is characterized by a peak in IL-6 one day before birth, followed by a peak of IL-10 and corticosterone three hours after delivery,

increasing the neuroinflammatory status in the hypothalamus and hippocampus during a critical narrow perinatal window. Thus, temporal elevations of inflammatory cytokines following the birth might have a physiological relevance for the neonatal developing brain ¹⁶⁷.

2.2.2 Feeding mode (Breastfeeding vs. Formula)

Although to a lesser extent than the birth delivery mode, breastfeeding also influences the assembly and maturation of the infant gut microbiota ¹⁵⁸. It is now established that breast-fed neonates have a much more diversified microbiota than formula-fed infants ¹⁷¹. More specifically, there is a more rapid and dense bacterial colonization of *Bifidobacteria*, *Clostridium* and *Bacteroides* in breastfed children. By contrast, there are higher proportions of *Escherichia coli*, *Clostridium difficile* or *Bacteroides fragilis* bacteria in the gut microbiota of formula-fed children, which can be at the origin of serious infections when these bacterial strains are too abundant in the GI tract ^{116,171}. In addition, human milk contains unique bioactive oligosaccharides, called human milk oligosaccharides (HMOs), that are the major dietary substrates for *Bifidobacteria* species, ¹⁷². Besides oligosaccharides, maternal milk contains immunoglobulins (e.g., sIgA), IgG and IgM, as well as various cytokines and growth factors, that are pivotal for the infant's developing immune system and general growth, especially before the transition to a more complex diet ^{116,127,173}. Also, c-section babies gut microbiota in the neonatal period are more enriched in opportunistic pathogens in the absence of breastfeeding ¹⁵⁸, suggesting that breastfeeding might be pivotal for the immune protection of the infant against pathogen infection in early life. In humans, exclusively breastfed infants show increases in IL-17 cytokine production, subsequently promoting T reg cells expansion compared to formula-fed infants and overall, breastmilk controls the immune inflammatory reactivity to non-maternal antigens ¹⁷⁴. Importantly, the beneficial effects of breastfeeding on the development of the regulatory immunity and the tolerogenic response towards microbial antigens may be mediated by SCFAs, as breastfed infant gut microbiota display higher relative abundances of SCFA-producing bacteria (e.g., *Veillonella* and *Gemella*) ¹⁷⁴. In turn, this might reduce the risk for developing auto-immune and/or inflammatory diseases later in life.

Breastfeeding was also shown to be beneficial for the development of the microbiota-gut-brain axis. Besides the numerous beneficial molecules and cells that human breastmilk harbors, there are also neurotrophic factors, such as glial cell-line-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF), and cytokines that can promote the infant ENS development ¹⁷⁵. Some other milk-derived molecules and microbes were also shown to influence the gut microbiota composition and the brain response to early life stress, since

maternal separation-induced stress in rats is dampened by the supplementation with milk fat globule membrane (MFGM) and/or prebiotics at weaning¹⁷⁶. Both MFGM and HMOs promote cognitive and socio-emotional development in breastfed rat pups^{176,177}. Interestingly, MFGM alone or combined to prebiotics improves cognitive functions of non-stressed weaned rats in parallel with changes in gut communities^{176,177}, suggesting that milk-derived components such as MFGM and HMOs act as prebiotics for the colonizing gut microbes and might be essential for cognitive development in healthy rat pups. Both MFGM and prebiotic mixture also compensate HPA axis stress hyperreactivity, which is reflected by decreased glucocorticoid receptor gene expression in the hippocampus and decreased levels of plasma corticosterone¹⁷⁶. In human infants, breastfeeding was associated to better child cognitive development, and the longer the breastfeeding is (*e.g.*, as opposed to partial breastfeeding and formula feeding) during the lactation period, the better cognitive tasks scores were in 5-year-old children¹⁷⁸.

Overall, these studies highlight the importance of breastmilk components and microbes for the proper development of infant's health, including its beneficial effects for the development of the regulatory immune system and the establishment of brain function and behaviors. These effects may be related to the promotion of milk components and/or breastmilk maternal microbes to a healthy and balanced gut microbiota in the infant. Although today's infant formula tends to be enriched in probiotics (*e.g.*, *Bifidobacteria*, *Lactobacillus*) and *Bifidobacteria*-promoting prebiotics, the exact period, dose and duration for which the exposure to these bacteria and prebiotics are optimal remains to be determined. In addition, other confounding factors such as maternal stress and diet, genetic background as well as environmental factors (*e.g.*, exposure to pollution, pets, etc.) can impact the microbial colonization process of the infant gut and subsequent health¹¹⁶.

2.2.3 Maternal infections and stress

Several rodent models of early life adversity provided the evidence that perinatal adverse events are detrimental for the infant development. In the prenatal period, maternal stress disturbs both the vaginal and intestinal microbiomes during gestation, which alters the offspring gut microbiota assembly and maturation^{66,122,179–181}. Importantly, this leads to a generalized pro-inflammatory state, transferred to the fetal brain via the placenta¹⁸⁰, and GI functional defects in early postnatal life^{176,179–181}. These GI issues are characterized by increased visceral pain¹⁷⁶ and gut leakiness⁶⁶, facilitating inflammation and transfer of microbial components. Stress-induced inflammation in the prenatal period is also observed in the brain of offspring later in life, and interferes with the development of key neurotransmitter signaling pathways such as

the serotonergic system¹⁸¹ and central neurotrophic factor levels (e.g., BDNF)¹⁸⁰. Consequently, the prenatally-stressed offspring show HPA axis stress hyperreactivity, cognitive defects^{176,179} and alterations in socio-emotional behaviors^{176,180–182}. Likewise, a pioneering study from *Hsiao and colleagues*⁶⁶ revealed that microbiota-derived metabolites, such as the 4-EPS, might mediate the physiological and neurobehavioral defects of perinatal adverse events⁶⁶. As maternal immune activation exacerbates the offspring's immune system and inflammatory responses^{66,91,183}, the gut-immune-brain signaling might be another pathway by which these neurobehavioral outcomes are observed. Indeed, increased numbers of Th17 cells and T regs in the MLNs are associated with somatosensory cortex impairments and were found to be dependent on the maternal IL-17a pathway^{91,183}, while microglia activation, IL-1 β and IL-10 central levels are increased in the ventral hippocampus in this mouse model^{91,184}. Nevertheless, alterations in the microbiota-gut-brain axis of offspring exposed perinatally to stress or infection can be reversed by early exposure to prebiotics¹⁷⁶ or probiotics, such as *Bacteroides fragilis*⁶⁶. These findings mirror the symptomatology of autism spectrum disorders (ASD)¹⁸⁵ since prenatal adverse events impact the brain and behavior in a sex-dependent manner^{180–182}. Indeed, early life adverse events mostly affect the cortical development in males and associated social deficits¹⁸¹, whereas female offspring show vulnerability in the amygdala and are more prone to increased compulsive and anxiety-like behaviors^{180,182}. These sex-specific alterations are reflected by sex-dependent transcriptional changes in the medial prefrontal cortex¹⁸², an important brain regions involved in the regulation of stress coping and related socio-emotional behaviors. In humans, a very recent study¹⁸⁶ found a moderate positive correlation between salivary cortisol and the gut microbiota composition in early infancy. This clinical study would thus confirm the pre-clinical observations and the potential link between maternal adverse events, the establishing gut microbiota and the development of the HPA stress axis.

2.2.4 Maternal diet

Likewise, the maternal diet and metabolic status are of importance for the development of the infant's gut microbiota and health. Maternal obesity in mice, using the high-fat diet regimen, leads to significant shifts in the composition of the early and adult gut microbiota in offspring^{187,188}, and is characterized by enriched bacterial populations promoting fatty acid metabolism, gluconeogenesis and mucin degradation in the maternal gut microbiome¹⁸⁹. Similarly, gestational diabetes mellitus is associated with a significant shift in the relative bacterial abundance in the maternal gut, vaginal and oral microbiomes, which is reflected in the

newborn's microbiota ¹⁹⁰. Interestingly, this last study highlights that maternal diabetes during pregnancy increases the presence of certain viruses in the neonatal gut microbiota, indicating that the maternal metabolic status in the perinatal period may impact the pathogenic status of the newborn's gut microbiota ¹⁹⁰. In humans, children born from obese mothers display significant differences in their fecal microbiota composition, characterized by differences in the relative abundance of *Faecalibacterium spp.*, *Eubacterium spp.*, *Oscillibacter spp.*, and *Blautia spp.*, that are associated with the regulation of body weight and diet ¹⁹¹. Besides the induction of a long-lasting gut dysbiosis in offspring, maternal high-fat diet leads to significant impairments in the intestinal barrier proliferation and integrity, low-grade inflammation, and higher body weight gain in weaned offspring, which is typical of metabolic disorders. These detrimental effects are maintained in adulthood by the exacerbation of experimentally-induced colitis in offspring ¹⁹², suggesting that maternal metabolic dysregulation in obesity is transmitted to the offspring and has enduring effects on his metabolic and inflammatory statuses.

These enduring effects also impact the developing brain and behavior, especially in the hypothalamus, a central region for the regulation of food intake, appetite but also stress regulation and socio-emotional behaviors ^{187,188}, as well as the mesolimbic reward system ¹⁸⁷. Likewise, females transplanted with an obesogenic gut microbiota before conception, not only show microbial alterations in the offspring's gut microbiome, but leads to alterations in early social communication, increased compulsive and anxiety-like behaviors, only in male offspring ¹⁸⁸, which is reminiscent of both NDD prevalence and symptomatology. Furthermore, the lack of microbiota in mouse dams induces an obesity-prone phenotype in the adult offspring, partly because of the dysregulated development of metabolic tissues resulting in excessive insulin levels and retardation of pancreatic β -cell differentiation as well as EEC differentiation. *Kimura and colleagues* ¹⁹³ have recently found that maternal-derived SCFAs are essential for the protection against HFD-induced metabolic syndrome and obesity later in life in the offspring. Maternal SCFAs are translocated to the embryos and sensed through GPR41 in the sympathetic nerve and GPR43 in the pancreas and intestinal tract ¹⁹³, thus highlighting that a healthy gut microbiota in the mother is paramount for the proper development of many bodily functions in the offspring. It also shows that metabolic dysregulation of the maternal microbiota might have detrimental effects later in life. The findings of this seminal study are depicted in the **Figure 7** below.

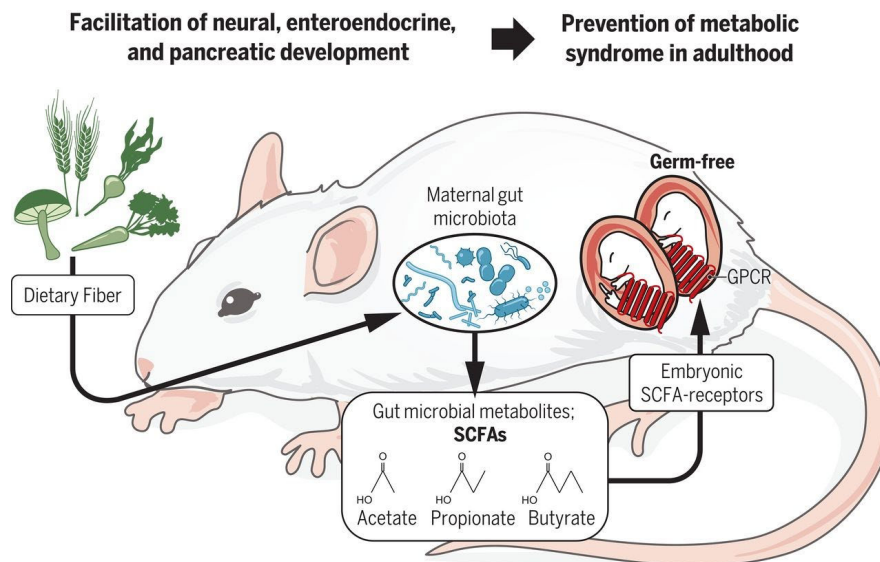


Figure 7. Beneficial effects of SCFAs derived from maternal high-fiber diet on offspring obesity predisposition are mediated by embryonic SCFAs receptors. In their pioneer work, Kimura and colleagues ¹⁹³ demonstrated that microbial-derived SCFAs (*i.e.*, acetate, propionate and butyrate) from the maternal gut microbiota can be translocated to the fetus through the placenta. Interestingly, maternal SCFAs are recognized by its receptors, GPP41 and GPR43, in the embryo, which potentiates the development of neural, pancreatic β cells and GLP-1-producing EECs. Thus, maternal SCFAs in the prenatal period are beneficial for the development of the metabolic function, and protects against the development of the metabolic syndrome later in adulthood. Figure from *Kimura et al., 2020*.

Altogether, these reports suggest that maternal diet before, during and after gestation is a key factor for a proper assembly and maturation of the offspring. As gut microbes influence many functions including the gut and the brain, notably through microbial metabolites such as SCFAs, maternal diet is critical for the offspring's metabolism and neurobehavior later in life ¹⁹³.

2.2.5 Antibiotic exposure

Although some recommendations have been made, at least in France ¹⁹⁴, to limit the use of antibiotics at this period, more than 50% of children is exposed at least once to an antibiotic during the first year of life ¹⁹⁵, and are thus considered as the most prevalent medication in early life. Their intensive use in the perinatal period is mainly to prevent life-threatening infections, both in infants and mothers ¹⁹⁴. For instance, as premature births often involves c-section delivery, broad-spectrum antibiotics are widely used as prophylaxis ¹⁹⁴. However, prenatal or perinatal antibiotic exposure is associated with decreased levels of *Bacteroidetes* and *Actinobacteria* phyla and increased *Proteobacteria*, as well as decreased microbial diversity in the infant gut microbiota ¹⁹⁶, that can persist until three years of age ⁴⁹. Of note, the low

abundance of *Bacteroides* in the gut microbiomes of babies delivered by c-section is strongly associated with intrapartum antibiotics used as prophylaxis ¹⁵⁸.

Increasing evidence demonstrate that antibiotic-induced disturbances of the gut microbiota have detrimental consequences for health and disease progression later in life. Broad-spectrum antibiotics exposure delays the maturation of the developing immune system and thus the protection against pathogens ¹¹⁶. In the pre-term infant mouse ^{196,197}, maternal antibiotic exposure (*e.g.*, three last days of pregnancy until the first two weeks of life) impairs the CD8+ T effector cells responsiveness to viral infection in pups ¹⁹⁷, which likely results from an incomplete TLR signaling stimulation in the immune system. Furthermore, prenatal and/or perinatal antibiotic exposure alters the levels of follicular B lymphocytes and CD4+ T cells in the spleen of mouse pups ¹⁹⁶ and low-dose penicillin in the perinatal period leads to decreased levels of T helper cells and antimicrobial peptides in the ileum of adult offspring ¹⁹⁸. Importantly, this delay in the immune system facilitates inflammation, life-threatening infections such as neonatal necrotizing enterocolitis ¹⁹⁹, and increases the risks of developing auto-immune disorders and allergies, such as asthma ^{200–203}.

Early life antibiotic exposure is also detrimental for the intestinal structure and function ^{149,204–207}. Exposure to vancomycin in neonatal life is sufficient to disturb the establishing gut microbiota, as well as the structure and the motility of colonic myenteric neurons of exposed mice later in life ²⁰⁸. Also, antibiotic exposure leads to a decreased number of colonic 5-HT mucosal cells, suggesting that the colonizing gut microbial populations during the early postnatal period is required for the regulation of the ENS function via its interaction with host 5-HT signaling pathway ²⁰⁹. The juvenile period appears to be also crucial to the full development of the ENS as antibiotic-induced dysbiosis during this specific time window leads to abnormal structure and function of the ENS, including alterations in GI transit and bile acid metabolism, disturbances of myenteric neurochemistry and morphology, as well as defects in the myenteric glia ¹⁴⁹. Similarly, maternal antibiotic exposure in rat dams during the perinatal period leads to increased gut permeability and growth defects across the whole GI tract of 2-weeks-old pups, and is associated with increased levels of *Enterobacteriaceae* (including *E. coli*) in their gut microbiota ²⁰⁴, showing that disturbances of the neonatal microbial colonization has direct effects on the developing GI tract. Interestingly, prenatal antibiotic exposure in mice leads to similar intestinal structure defects, notably ileal atrophy ¹⁹⁸, a decrease in Goblet cells numbers and tight junction protein expression, as well as an increased susceptibility for gut inflammation in mouse neonates ²¹⁰. In humans, antibiotic exposure in the

first year of life is positively associated with increased risk to develop celiac disease ²⁰⁶, a chronic auto-immune condition that involves a strong gut inflammation and destruction of small intestinal villi when gluten is ingested. In rats, early exposure to vancomycin or a cocktail of antibiotic leads to increased susceptibility to abdominal pain later in life in male subjects, but not females ²⁰⁵, suggesting a sex-dependent effect of early postnatal antibiotic exposure on GI pain sensitivity. Likewise, gut motility and the ENS are impaired significantly when broad-spectrum antibiotics are administered either in the neonatal ²⁰⁹ or the juvenile period ¹⁴⁹, which again highlights the importance of these postnatal time windows in mice for the proper development of the ENS. As gut function is impaired following early life antibiotic exposure, the digestive and metabolic systems are also impaired in that context. Pioneer work from Martin Blaser's laboratory has shown that early life exposure to low-dose of penicillin in the perinatal period induces metabolic changes later in adulthood ¹⁹⁸. Importantly, low-dose penicillin exposure in that period aggravates the effects of high-fat diet on hepatic function, metabolic hormone levels and accumulation of visceral fat in mice ¹⁹⁸, thus suggesting that subtherapeutic doses of antibiotics and its effects on the gut microbiota composition in early life mediates high-fat diet-induced obesity later in adulthood, at least in mice ¹⁹⁸. Moreover, disturbing the mouse microbial colonization during small intervals in the perinatal period, that mimics antibiotic exposure in humans during early life, increased the severity of type I diabetes, that is associated with gut microbiota disturbances ²⁰³. Thus, early life antibiotic exposure is detrimental for the development of a healthy metabolism and promotes the development of long-lasting metabolic diseases such as obesity or diabetes ^{198,203,211,212}. It might be also relevant to humans as a clinical study ²¹² reports that infant antibiotic exposure is correlated to increased risk for obesity and childhood adiposity.

Altogether, these observations indicate that early life antibiotic exposure, either prenatally, postnatally or both, impacts the microbial colonization and maturation of the infant gut, which ultimately leads to an immature immune system, and potentially long-lasting GI dysfunction and metabolic disturbances. In addition, this leads to increased risks for debilitating diseases such as type I diabetes, obesity or inflammatory bowel diseases. Alarming, the detrimental effects of early life antibiotics can extend until the later life, as aged mice exposed to ampicillin and neomycin in the perinatal period show an increased insulin sensitivity, increased inflammatory levels and a reduced lifespan ²¹³.

Most importantly, increasing evidence was brought in the microbiota-gut-brain axis field for the impact of early life antibiotics on brain function and behavior, including the development

of the HPA axis and stress-related and socio-emotional behaviors, brain permeability and function^{214–220}, which is further detailed in the following section.

2.3 Influence of the gut microbiota on neurodevelopmental processes

It is now widely established that the development of brain function and behavior is critically occurring during the first 1000 days of life in infants, that corresponds to the perinatal window between conception and the first three years of age²²¹. This specific time window also parallels the time during which the maternal microbiota and the microbial colonization influences infant's health (see **Figure 8**), including various brain developmental processes.

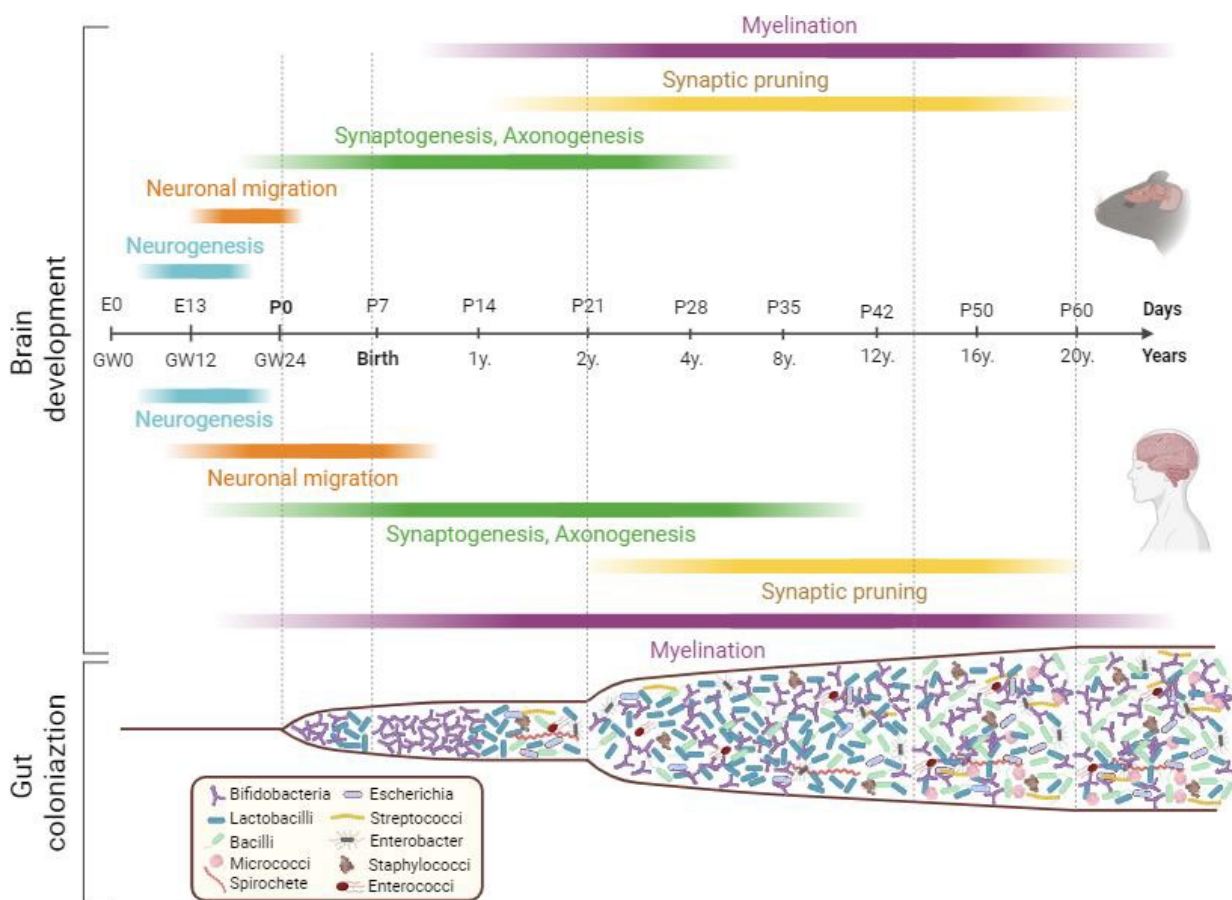


Figure 8. Brain developmental milestones in both mice and humans, and parallel gut colonization process. Timing of development of the main brain developmental processes in mice (upper part), and the timing equivalence in humans (middle part), aligned with the evolution of the gut microbiota (lower part), presented with the main commensal bacterial species in the gastrointestinal tract over time. Adapted from Zeiss *et al.* (2021)²²²; Khodosevich and Sellgren (2023)²²³ and Kalbermatter *et al.* (2021)¹²⁹. Created with BioRender.

2.3.1 Neurogenesis and neuronal migration

Neurogenesis corresponds to the physiological phenomenon whereby neural stem cells and progenitor cells migrate and differentiate to new functional neurons^{224,225}. Indeed, neurogenesis is a complex and sequential process driven by multiple molecular elements, including growth hormones and transcription factors, neurotransmitters and metabolites²²⁶. Moreover, a wide range of studies^{96,164,165,224,227–229} are now showing that gut microbes, their metabolites or their components participate to the establishment of neurogenesis, axonogenesis and neuronal cell death both in early life and during adulthood.

During gestation, maternal microbial metabolites and bacterial-derived components from the maternal microbiota can cross the placental barrier to reach the fetal brain and contribute to neurogenesis and axonal growth^{227,228}. A pivotal study²²⁸ showed that maternal microbes modulate the bioavailability of produced metabolites and promote axonal growth and elongation in the fetal brain, particularly in the thalamus, cortex, striatum and hypothalamus. As these brain regions regulate sensorimotor functions, suppression of the maternal microbiota (*i.e.*, complete absence of microbes or antibiotic depletion in the mother) alters partially the tactile sensory responses later in adulthood. At the molecular level, this important study revealed that commensal bacteria belonging to the *Clostridia* family, are sufficient to induce axonal growth and elongation in the thalamus, and would very likely signal through microbial metabolic by-products (see **Figure 9** below)²²⁸.

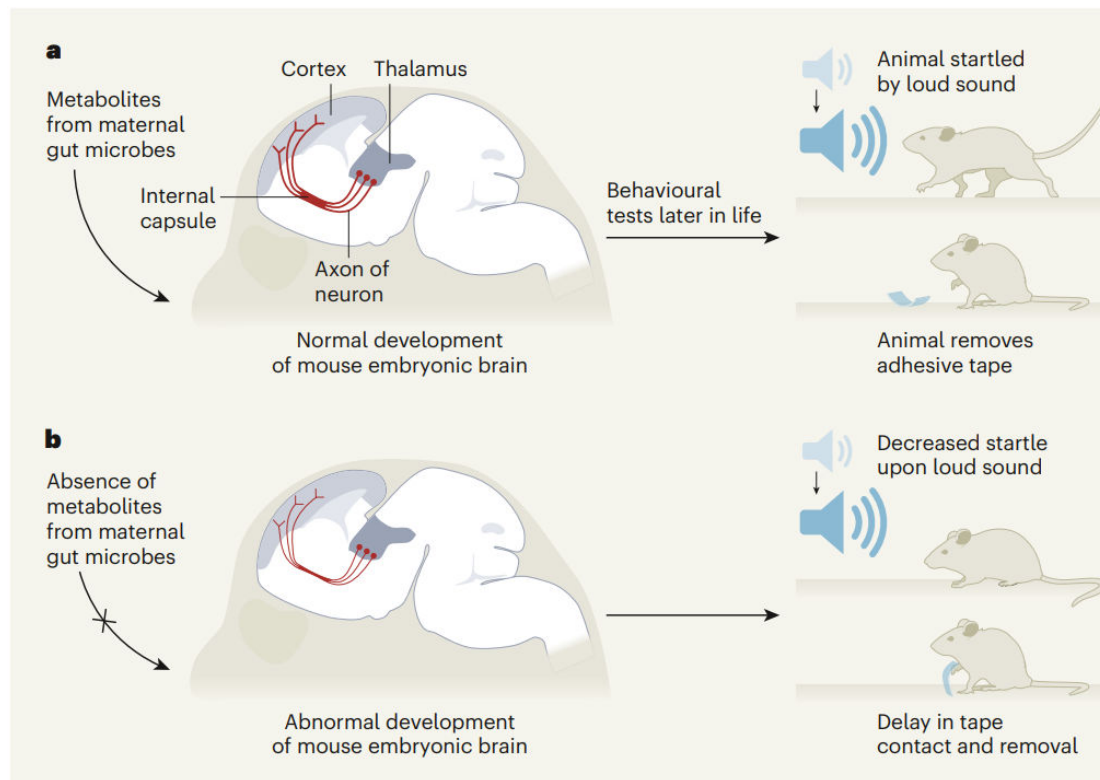


Figure 9. Maternal microbial metabolites influence prenatal brain wiring and related sensorimotor behaviors. In the study of *Vuong et al.*²²⁸, evidence was provided about (a) maternal microbial by-products translocating to the fetal brain, contributing to the normal development thalamocortical neuronal connections in the fetus, relayed by an axonal bundle in the internal capsule. At the behavioral level, this particular neuronal network controls sensorimotor functions, such as reactions to a loud sound (known as the ‘startling response’) or tactile stimuli (e.g., paw removal in the adhesive tape test). However, (b) absence or depletion of the maternal gut microbiota during the prenatal period leads to the absence of these maternal gut metabolites, leading to lower axonal density in the thalamo-internal capsule-cortical axis in the fetal brain. Consequently, sensorimotor delays are observed in the developed offspring, including decreased startling response and delayed tape contact and paw removal in the adhesive tape test. Figure from *Meckel and Kiraly (2020)*²³⁰.

Postnatally, colonizing gut microbes contribute to the ongoing differentiation and maturation of neurons. Disruption of microbial colonization with broad-spectrum antibiotics during the postnatal period in mice leads to impaired adult hippocampal neurogenesis^{231,232} and dendritic spine density, that results in impaired neuronal plasticity and behavioral deficits. Further molecular analyses in this study have demonstrated that a bacterial-derived metabolite, the 4-methylphenol, detected in more elevated levels in the serum of antibiotic-treated mice, might mediate these hippocampal and behavioral impairments²³¹. Commensal gut microbes might

also influence neurogenesis in an indirect manner through the immune system, as it was shown that adult hippocampal neurogenesis might depend on brain-resident Ly6C^{hi} monocytes, signaling to hippocampal stem and progenitor cells⁹⁶. Another study¹⁷⁷ has shown that stress-induced reduction in hippocampal neuro-proliferation is rescued with HMOs, that promotes the growth of commensal bacteria. In turn, this corrects the alterations in social and anxiety-like behavior observed in stressed animals and indicate that promoting bacterial growth with prebiotics received from the maternal milk may also be neuroprotective in the developing infant. Importantly, neuronal cell death is a complementary neurodevelopmental process that is programmed around birth²²⁵ for the maturation of proper functional neurons later in life, and also prevents neuronal excitotoxicity²³³. Thus, this process is paramount for later brain wiring, neuronal functioning and behavior. Interesting findings from *Castillo-Ruiz and colleagues*¹⁶⁵ have shown that the disruption of the microbial colonization at birth, caused by c-section, may affect the programmed neuronal cell death process. Indeed, c-section-born pups display higher numbers of neurons between birth and the three postnatal days in many brain regions, including the hypothalamus and the hippocampus. At weaning, the c-section delivery mode results in fewer vasopressin neurons, involved in the regulation of the stress response and socio-emotional behaviors, leading to neonatal communication defects¹⁶⁵. Altogether, this suggests that cues from early microbial colonizers are necessary to control the balance of neuronal density and thus the development of cognitive and affective behavioral abilities.

2.3.2 Synaptic development, dendritic growth and myelination

In parallel to the molecular determination of neuronal fate, synaptic development, dendritic growth and axonal myelination are initiated in the developing brain to establish functional and mature neuronal connections, in turn shaping the brain synaptic plasticity (see **Figure 8**). Similar to neurogenesis, these neural processes start *in utero* and continues until the postnatal period, in which they are refined depending on genetic and environmental cues across life^{77,234}. Moreover, they involve many cell types (*e.g.*, neurons, astrocytes, oligodendrocytes, microglia) and molecular events (*e.g.*, neurotrophins signaling) to be initiated²²⁴. A study using GF mice²³⁵ has revealed that the absence of microbiota leads to abnormal dendritic morphology and thus, deficits in synapse formation. Adult GF mice indeed show dendritic hypertrophy in inhibitory interneurons and excitatory pyramidal neurons of the basolateral amygdala and the hippocampus. Notably, the numbers of spines in these neuronal subtypes are affected by the absence of microbial cues²³⁵, resulting in increased synaptic connectivity in these brain regions and a dysregulated balance between inhibitory/excitatory neuronal stimuli. These findings are

confirmed in the brains of c-section-delivered mouse pups, that show differential trajectories in early life microbial colonization compared to naturally delivered mice ^{164,166}. Indeed, c-section decreases dendritic arborization in pyramidal neurons of the prefrontal cortex (PFC) and hippocampus ¹⁶⁴, and alters the hippocampal transcriptome later in young adult mice ¹⁶⁶. Consequently, this has enduring neurodevelopmental outcomes on social and affective behaviors in mice, that are already visible in the early postnatal period ¹⁶⁶. Moreover, exposure to *Bifidobacterium breve* or *B. breve*-targeted prebiotic (*i.e.*, a mixture of fructo-oligosaccharides and galacto-oligosaccharides) partially restores social cognition, maternal attachment and early life social communication impairments ¹⁶⁶. Thus, microbial colonization during early life, especially with *Bifidobacteria* during the neonatal period, may be necessary for the establishment of dendritic arborization, and thus the formation of synapses, that will condition later brain functioning in limbic areas regulating mood and behavior.

Synaptic formation and remodeling are dependent on molecular cues such as neurotrophins (*e.g.*, BDNF) and synaptic proteins including synaptophysin (*i.e.*, a synaptic vesicle glycoprotein) and the postsynaptic density 95 protein (PSD-95) ²³⁶. Mice devoid of commensal microbes show decreased gene expression of BDNF in the basolateral amygdala ^{236,237}, hippocampus ^{62,236} and cortex ⁶²; while synaptophysin and PSD-95 are upregulated in the striatum of these mice ²³⁶. Likewise, differential gene expression of canonical pathways regulating synaptic plasticity and synaptic long-term potentiation is observed in GF mice ²³⁶, indicating that microbial cues are necessary for the development of mood and affective behaviors, including anxiety-like behavior ²³⁶, locomotor activity ²³⁶ and social preference ²³⁷.

Concomitant to neurogenesis, dendritic and synaptic development, the myelination process occurs in late prenatal life until infancy ^{77,225}, and more specifically between the last trimester of pregnancy (*i.e.*, 32 prenatal weeks) and the first 2 postnatal years in the human infant ²²¹ (see **Figure 8**). Myelination constitutes an important developmental milestone for neuronal connectivity as it speeds up neurotransmission along axons, thanks to a surrounding conductive lipid-rich envelope, called the myelin sheath ²³⁸. Oligodendrocytes are glial cells that produce myelin around neurons ²³⁸ and start proliferating in the prenatal period and, unlike astrocytes and microglia, their proliferation continues until the adult age ²³⁴. Pioneering work ²³⁹ using the GF mouse model showed that the absence of microbiota is detrimental for the myelination process. This study demonstrates indeed that myelin-related genes are upregulated in the PFC of GF mice, compared to conventionally-colonized counterparts, and are reflected phenotypically with increased myelin sheath thickness, indicating that commensal microbes

send signals to oligodendrocytes of the PFC to control the production of the myelin sheath around axons. Importantly, colonization of GF mice post-weaning does not rescue defects in the PFC myelin sheath²³⁹, suggesting that there is a critical time window during early life for the proper formation of axonal myelination. These results were confirmed in mice whose microbiota is depleted with antibiotics in the neonatal life, as they display hypermyelination in the PFC, associated with cognitive and anxiety-like behavioral impairments, and GI impairments²³². Interestingly, rescuing the antibiotic-induced gut dysbiosis with microbial-derived butyrate specifically at weaning is sufficient to rescue these microbiota-gut-brain-behavior impairments caused by neonatal antibiotic exposure²³². Recently, a seminal study²⁴⁰ has evidenced that colonization in mice with bacteria metabolizing tyrosine into the bioactive metabolites 4-ethylphenol (4-EP) and 4-ethylphenyl sulfate (4-EPS) leads to changes in neuronal connectivity by directly influencing the maturation of oligodendrocytes. In particular, 4-EP can cross the BBB and affects the myelination patterns in multiple brain regions, including the hypothalamus, the bed nucleus of the stria terminalis (BNST), the hippocampus and the amygdala, that are all pivotal for the regulation of affective behaviors. Expectedly, demyelination by these bacterial metabolites is associated with increased anxiety-like and compulsive-like behaviors²⁴⁰. Similarly, myelination defects in a socially-despaired mouse model seems to be driven, at least in part, by the microbial metabolite *p*-cresol, deriving from metabolization of tyrosine by *Clostridiales* bacterial species (*i.e.*, *Lachnospiraceae* and *Ruminococcaceae*) in the gut microbiota²⁴¹. Indeed, *p*-cresol results in hypomyelination in the medial PFC and amygdala and diminishes the numbers of oligodendrocytes *in vitro*²⁴¹, indicating again that microbial metabolites are involved in the development of the myelination process in the medial PFC and amygdala, two key brain areas implicated in the regulation of mood and affective behaviors. In conclusion, gut bacteria are able to synthesize neuroactive metabolites, such as 4-EP, 4-EPS, *p*-cresol or SCFAs, that would directly act on neural processes such myelination, dendritic arborization, modeling synaptic connections. As a consequence, these metabolites produced by the gut microbiota during critical period of neurodevelopment, especially in the pre-weaning period, influences the shaping of neuronal networks in brain regions regulating cognition, mood and socio-emotional behaviors.

2.3.3 Blood brain barrier integrity

The blood-brain barrier (BBB) is the physiological barrier between the brain and its blood vessels. The BBB is composed of different cell types and molecular components (*e.g.*, endothelial cells, pericytes, glia cells, neurons and extracellular matrix) that provide a

protection to the CNS from pathogens and certain toxins. Tight junction proteins are important components of the BBB because they ensure a physical cohesion between endothelial cells and limits the paracellular diffusion through the BBB²⁴². The BBB is also significantly influenced by the gut microbiota, especially in the perinatal life. In a landmark study, *Braniste and colleagues*⁹⁸ have shown that absence of the gut microbiota in GF mice led to disruption of the BBB in prenatal life that persisted until adulthood. This disruption is due to decreased gene expression of tight junction proteins in the brain. However, colonization of GF mice with SCFA-producing bacteria and/or SCFAs alone, leads to a partial rescue of tight junction protein expression in the same brain areas⁹⁸. Similarly, gut microbiota depletion with broad-spectrum antibiotics in adult mice leads to a similar effect on BBB permeability to those observed in GF mice²⁴³. The authors showed that transplantation of monocytes into the bone marrow after antibiotic-induced depletion of the gut microbiota facilitated the infiltration of bone-marrow-derived monocytes into the brain. Ultimately, peripheral inflammation induced by antibiotics, which is characterized by increasing levels of pro-inflammatory molecules (*e.g.*, IL-1 β , IL-6 or TNF α) in the blood circulation, also facilitated BBB permeability and infiltration of immune cells into the brain. Altogether, these findings indicate that the gut microbiota contributes to the normal development of BBB integrity, by acting on the tight junction protein complex of BBB endothelial cells, notably through microbial-derived SCFAs. Further, fecal microbiota transplant to these mice restores, at least partially, impairments of the BBB permeability by upregulating tight junction protein gene expression, further highlighting the role of microbial-derived metabolites in the regulation of BBB integrity during neurodevelopment.

2.3.4 Microglia maturation

Microglia represents the CNS-resident immune cells which function is to maintain immune homeostasis and to participate to the control of inflammation within the CNS. Moreover, microglial cells contribute to many neurodevelopmental processes^{77,224} such as neuronal proliferation and differentiation, neural processes formation and the establishment of synapses²³⁴. As such, microglia is a pivotal actor in brain development and behavior. Although deriving from embryonic yolk sac macrophages, microglia genesis peaks at the first year of life and extends until the childhood period²³⁴, paralleling the normal host microbiota colonization²²⁵.

As for the peripheral system, GF mice display an immature CNS immune system, notably with the alteration of microglial gene expression profile and underdeveloped microglia^{95,244,245}. Indeed, microglial cells from GF mice or antibiotic-depleted mice show morphological defects, such as increased dendritic length and cell volume, as well as decreased cytokine response

following LPS-induced or viral infection⁹⁵, indicating the importance of microbial cues for host microglial development. Commensal microbes very likely program microglial developmental trajectories already in the prenatal period²⁴⁵. Microglia start proliferating in the fetal brain, but lack of microbiota leads to over-proliferation of microglia in the brains of GF fetuses. Furthermore, absence of microbiota is shown to dampen the regulation of microglia proliferation in a sex- and time-dependent manner. Male GF offspring microglia seems to be more vulnerable to the lack of microbiota in fetal life compared to female GF fetuses, while female GF adult's microglial function is more sensitive to microbes than males at the same age. Thus, there are sex- and time-dependent changes in microglial-related and immune-related genes during fetal life related to epigenetic modifications by maternal microbial cues²⁴⁵.

In the postnatal period, conventionalization of the gut microbiota with *Bifidobacteria* rescues microglial numbers and their ramified morphology in the cerebellum²⁴⁴. Likewise, gene markers of microglia phagocytosis are upregulated in the cerebellum at P4 following *Bifidobacteria* exposure²⁴⁴. Thus, *Bifidobacteria* colonizing the gut in the early postnatal period would be responsible, at least partially, of the postnatal shaping of host microglial populations as well as the microglial morphology, inflammatory and phagocytic functions. Interestingly, the beneficial effects of commensal *Bifidobacteria* on postnatal microglial development might be mediated by their production of SCFAs. Indeed, recolonization of antibiotic-exposed mice, or exposure to bacterial-derived SCFA butyrate in GF mice, rescues microglial morphological and functional defects. In line with the abovementioned studies, *Lynch and coworkers*²¹⁸ have shown that antibiotic-induced depletion of the gut microbiota in the postnatal period leads to severe microglial morphological defects, but they importantly reveal sequential development trajectories of microglia during the postnatal period²¹⁸. More specifically, the pre-weaning period seems to be crucial for the influence of gut microbes for gross morphology, such as the cell soma volume. By contrast, microglial cell branches would be more regulated towards the weaning and post-weaning periods, but not before²¹⁸. As microglia participates to synaptic refinement and the proper establishment of neuronal activity, some studies investigated the potential effect of neonatal microbial colonization on microglial function and synaptic connectivity. GF mouse neonates display overproduction of synaptic connections, lack of synaptic refinement, that are both reflected by the upregulation of synaptic-related genes (*i.e.*, immediate-early genes and *Bdnf* overexpression, that contribute to synaptic plasticity), and defects in microglial reactivity²⁴⁴. Nonetheless, continuous gavage with a mixture of four *Bifidobacteria* species (*i.e.*, *B. longum subsp. infantis*, *B. breve*, *B. bifidum*, and *B. dentium*) or

full conventionalization during the pre-weaning period leads to the downregulation of synaptic plasticity-related genes and upregulation of microglia-linked genes in the cerebellum, cortex and hippocampus of mouse neonates compared to GF counterparts ²⁴⁴. Moreover, *Bifidobacteria* exposure or full microbiota conventionalization after birth corrects synaptic pruning defects in GF neonates at P20. Similarly, the normal neuronal electrophysiological activity of cerebellar Purkinje cells is restored after *Bifidobacteria* colonization in P20 mouse pups. This important study thus highlights that *Bifidobacteria* commensals are essential in the first postnatal days in mice for the proper establishment of synaptic pruning in Purkinje cells dendritic trees, allowing cell development at weaning, and their overall contribution to the establishment of neuronal cell wiring ²⁴⁴. Whether this beneficial regulatory mechanism by *Bifidobacteria* is mediated by SCFAs remains to be elucidated.

Once mature microglial networks are established in the brain, commensal microbes still regulate the microglial response, and this beneficial influence seems to be mediated by the TLR4 signaling pathway ²⁴⁶. More precisely, perturbing the gut microbiota in adulthood, with antibiotics for instance, leads to a weak and insufficient microglial response to viral or LPS-induced infection, that is associated to altered expression of antigen presentation genes in the microglia of antibiotic-depleted animals. Consequently, perturbations of the microbiota, in the GF or the antibiotic treatment contexts for instance, worsens the viral-induced autoimmunity against neurons, leading to neurological damage ²⁴⁶. However, activation of microglia with TLR4 agonists in these mice significantly rescues these microglial impairments, suggesting that the microglial response against viral infection requires the presence of a ‘normal’ gut microbiota, thanks to the upregulation of antigen presentation genes in microglia and indicates a potential neuroprotective role of commensal microbes against viral-induced demyelination ²⁴⁶. Altogether, there is seminal evidence that microbial cues are essential for the development of microglia morphology and function during the perinatal period, in turn leading to balanced synaptic connectivity and a functional neuronal activity. As explained above, the fetal period seems to be critical for the regulation of microglial epigenetic programming by maternal microbes, which occurs in a sex-dependent manner, and might be at the origin of sex-biased neurodevelopmental or neuropsychiatric disorders later in life. In the postnatal period, there is a sequential morphological and functional development of microglia, that might be finely tuned by colonizing microbes, and probably by the predominant colonizing bacteria *Bifidobacteria* at this period of life. Moreover, microglial maturation by this bacterial taxa may be mediated by its metabolites, the SCFAs, but the exact mechanisms remain to be fully elucidated. In adult

life, gut microbes seem to still communicate with CNS-resident microglia to regulate their response towards inflammation and potential brain infection, mirroring the constant microbes-immune interactions in the periphery.

3 Gut microbes, neuropeptides, and socio-emotional behaviors

3.1 Neuropeptidergic systems regulating socio-emotional behaviors

3.1.1 Development and function of the oxytocin and the vasopressin systems

Location of oxytocin and vasopressin cells. Oxytocin (OXT) and vasopressin (also called arginine-vasopressin; AVP) are highly conserved endogenous peptides, that are closely related structurally and functionally^{247,248}. These nine amino acids peptides are synthesized by specific hypothalamic neurons that project to the posterior pituitary gland (or neurohypophysis), itself releasing OXT and AVP into the bloodstream at the periphery, together constituting the hypothalamo-neuro-hypophysial (HNS) axis (see **Figure 10**)^{247–250}. Two types of neuroendocrine cells express OXT and AVP, naming the magnocellular neurons (*i.e.*, with a large soma) and the parvocellular neurons (*i.e.*, with a relatively smaller soma)^{248,251–256}. Importantly, magnocellular neurons constitute the majority of the OXT and AVP neuronal populations and project to multiple brain areas^{248,251,252,257} and the posterior pituitary gland within the hypothalamo-neurohypophysial system (HNS)^{247,248,251,258}. By contrast, the few parvocellular neurons expressing either OXT or AVP within the same nuclei only project centrally, specifically to the midbrain and the hindbrain (*i.e.*, brain stem and spinal cord) (see **Figure 10**)^{253,254,259}. Along the HNS, OXT was first described for its modulation of milk ejection from the mammary glands, as well as uterine contractions during labor^{247,249,258,260}, while AVP is required for fluid homeostatic balance in kidneys^{261,262}. Importantly, a large body of evidence have demonstrated the pleiotropic roles of OXT and AVP in brain and behavior.

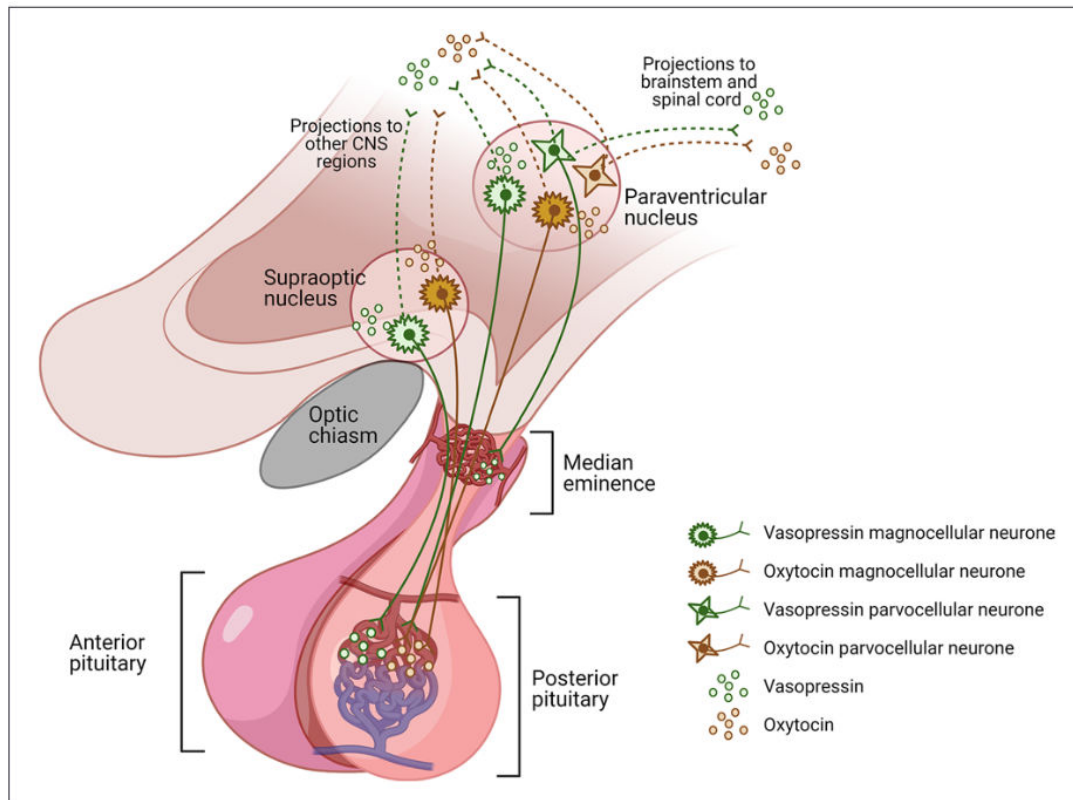


Figure 10. Schematic representation of the hypothalamo-neurohypophyseal system. Magnocellular neuroendocrine neurons of the OXT and AVP systems project axons from specific hypothalamic nuclei (e.g., the supraoptic nuclei or SON, and the paraventricular nuclei or PVN) to the posterior pituitary, from where the OXT and AVP neuropeptides are secreted into the bloodstream and periphery, altogether constituting the HNS. Magnocellular neuroendocrine OXT and AVP cells send long-range collaterals to the posterior pituitary through the median eminence. Along the HNS, OXT and AVP act as neurohormones, as they are released into the bloodstream and target distant organs, such as the uterus (OXT) or kidneys (AVP) for instance. Both magnocellular and parvocellular neurons project centrally, from the PVN and SON, and other OXT- and AVP-expressing hypothalamic nuclei, such as the accessory nuclei or the retrochiasmatic nuclei. Nonetheless, parvocellular neurons only project centrally and densely to the brainstem, spinal cord and midbrain areas, whereas magnocellular neurons send a complex range of projections to the forebrain, midbrain and hindbrain. Figure by *Bárez-López and colleagues (2023)* ²⁵⁸.

The neuropeptides OXT and AVP are synthesized in discrete hypothalamic nuclei, in majority in the bilateral paraventricular nuclei (PVN), supraoptic nuclei (SON), retrochiasmatic nuclei (RCH); and to a lesser extent, in some scattered neurons of the accessory nuclei (AN) ^{247–249,251,257,263–265}, the lateral hypothalamic area (LHA), the antero-dorsal preoptic nucleus (ADPN) and the ventromedial preoptic nucleus (VMPO) ^{251,263,266,267} (see **Figure 11**). Within

these nuclei, especially within the PVN and the SON, OXT and AVP neurons are located in distinct subdivisions. For instance, AVP cells are located in the postero-dorsal subdivisions of the PVN and SON, whereas OXT cells are found mostly in the antero-lateral parts^{248,263–265}. They are also present in extra-hypothalamic areas such as the bed nucleus of the stria terminalis (BNST)^{263,265} and some studies are even reporting OXT cells in the medial amygdala (MeA)^{265,266} (not depicted in **Figure 11**). Some hypothalamic nuclei show homogenous populations of either OXT or AVP neurons. As such, the antero-dorsal/antero-lateral preoptic nucleus, the BNST and the periventricular nucleus (PeVN) are primarily composed of OXT neurons whereas the suprachiasmatic nucleus (SCH) contains mostly AVP cells^{264,265}. Recent structural studies in the whole brain^{251,264–267} mapped the precise architecture of OXT and AVP connectome in the rodent adult brain, and revealed a complex network of projections.

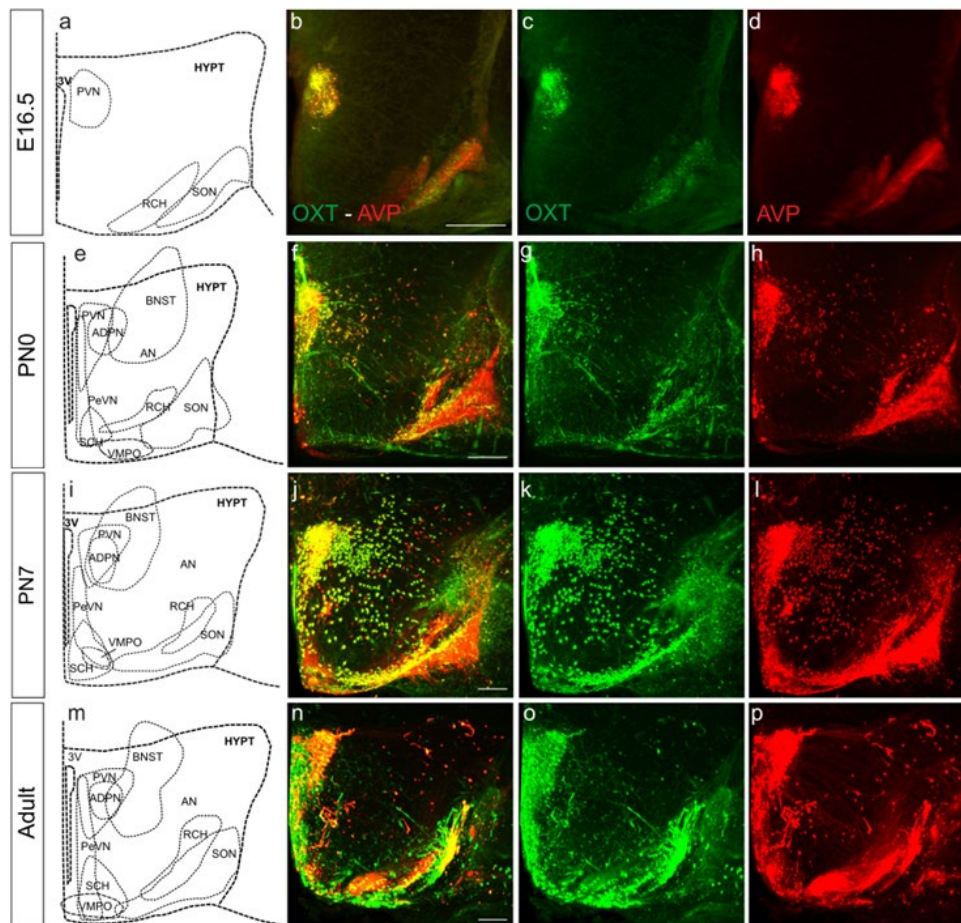


Figure 11. Spatiotemporal development of OXT and AVP neurons in the mouse hypothalamus. The data presented here were obtained by *Madrigal and Jurado (2020)*²⁶⁵ in the developing wild-type ICR outbred mouse by immunohistochemistry targeting OXT-Neurophysin I (in green) and AVP (in red) at several perinatal stages and in the coronal view: Embryonic day (E)16.5 (a-d), Postnatal day 0 (PN0, e-h), PN7 (i-l) and the adult (m-p), and associated schematic representations according to the brain

reference atlas of Franklin and Paxinos ²⁶⁸. Abbreviations: 3v third ventricle, ADPN anterodorsal preoptic nucleus, AN accessory nucleus, BNST bed nucleus of stria terminalis, PeVN periventricular nucleus, SCH suprachiasmatic nucleus, SON supraoptic nucleus, PVN paraventricular nucleus, RCH retrochiasmatic area, VMPO ventromedial preoptic nucleus. Scale bar: 200 μ m.

Ontogenesis of oxytocin and vasopressin neurons. The OXT and AVP systems are generated in the second half of gestation in both rodents and humans ^{247,249,260,269}. Nonetheless, the two neuropeptidergic systems display distinct temporal developmental trajectories, with a delay in the OXT system development compared to the AVP system in the perinatal period ^{255,260,264,270–272}. More particularly, OXT gene transcripts are detected from E15.5 in the developing PVN, and later at E18.5 in the SON; whereas AVP expression is already visible from E13.5 in the establishing SON and at E14.5 in the PVN of the mouse fetus ²⁷⁰. Moreover, the appearance of OXT mRNA is highly sex-dependent as the detection of OXT gene expression is delayed in the developing male brain compared to females (P2 vs. E16.5, respectively) ²⁷³. Although the mature OXT neuropeptide is detected at birth ^{247,255,260,274}, the AVP mature neuropeptide is detected as early as E16.5 ²⁶⁵, showing that AVP would be functional already in the late prenatal period, and hence earlier than OXT. The fact that mature OXT is only detectable at birth and that maternal OXT levels are very high during parturition leads to the hypothesis that maternal OXT is transferred to the newborn via the placenta and reaches the neonatal brain, ultimately facilitating the maturation of its OXT system (see **Figure 12** below) ²⁶⁰.

In the postnatal period, both OXT and AVP gene expression increases significantly, and the OXT cell development still shows a one-week delay compared to the AVP system ^{270,271}. In the first postnatal week, volume and numbers, as well as the fibers density of AVP neurons are higher than in the OXT system ^{265,272}. Both OXT and AVP cell bodies and fibers migrate from the AN to their final location, the SON and the PVN ^{265,272} (see **Figure 11**). Although the number of AVP neurons are similar between birth and adulthood ^{264,265}, the number of OXT neurons double between P0 and P14 in the PeVN and the ADPN ²⁶⁴, whereas the number of OXT neurons in the SON and neighboring ventromedial OXT-containing nuclei is already similar to adult levels at the same time ²⁶⁴. In addition, spatial organization of both neuropeptidergic cells types are different. From birth, OXT neurons migrate along the antero-posterior and the dorso-ventral axes in the PVN and the other anterior parts of the hypothalamus and stabilizes by the second postnatal week, whereas the spatial organization of AVP cells is already nearly established at this time of age compared to the OXT system ^{264,265}.

At the cellular level, electrophysiological and morphological maturation of OXT and AVP neurons is still incomplete in the first postnatal week, as they show an unstable membrane potential and immature electrical activity, as well as a limited number of neurites (*i.e.*, dendrites and axons), thus preventing their functional endocrine activity at this time of age ^{275,276}. Nonetheless, the second postnatal week in rodents is pivotal for the maturation of their morphological and electrophysiological properties (see **Figure 12**) ^{275–277}.

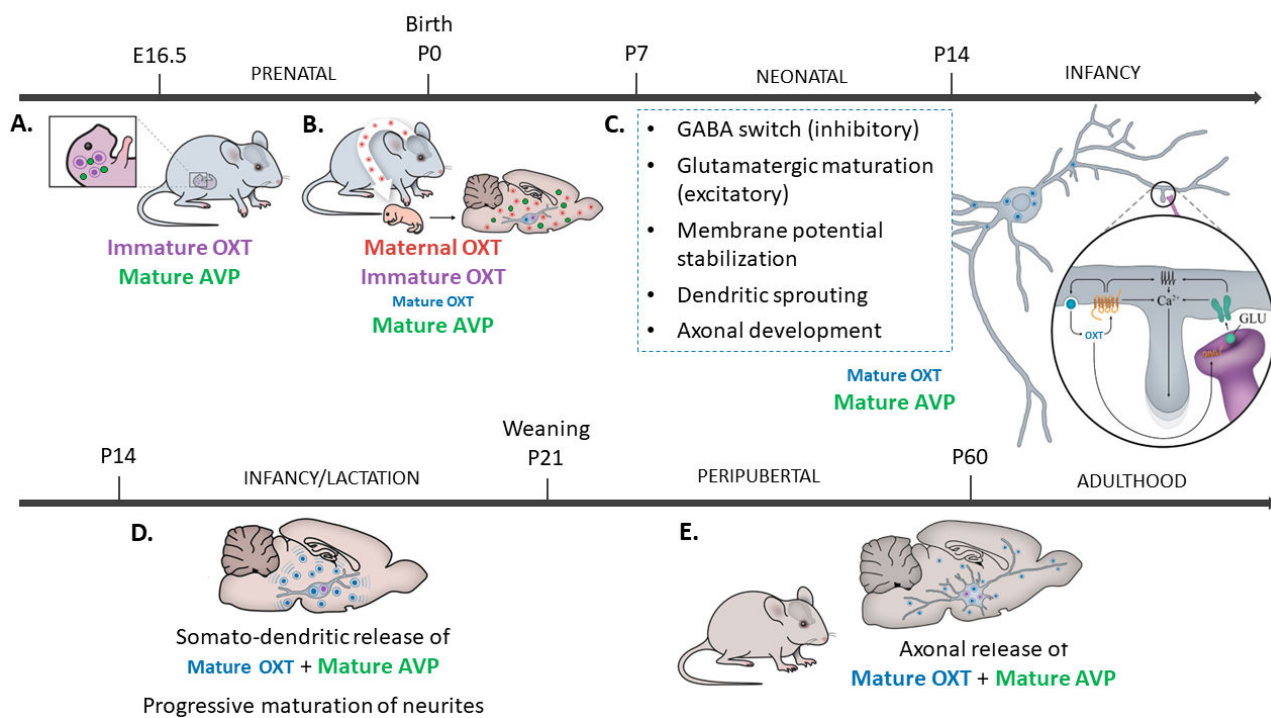


Figure 12. Maturation of OXT and AVP neurons electrophysiology and morphology. (A). Only immature forms of OXT are found in the prenatal brain whereas there would already exist mature forms of AVP neuropeptide. (B). During birth, blood OXT levels are extremely high in the mother and maternal OXT may reach the neonate via the placental barrier during delivery. It is hypothesized that maternal transfer of OXT may initiate the production of mature OXT in the offspring brain ^{255,260}. (C). During the second postnatal week, autocrine release of OXT (or AVP) mobilize intracellular calcium (Ca^{2+}) stores through the auto-activation of the OXTR (or AVPR1a), called ‘auto-control’ of OXT/AVP neurons. This leads to the activation of local glutamate release. Then, newly secreted glutamate binds to the same neuropeptidergic cell, which also activates the discharge of Ca^{2+} intracellular stores. Ultimately, Ca^{2+} release promotes dendritic sprouting, membrane potential stabilization and the excitatory glutamatergic signaling. In parallel, local OXT release promotes the shift from immature excitatory GABA to mature inhibitory GABA neurotransmission, therefore completing the maturation of the excitatory/inhibitory balance in the brain. (D). During the first postnatal weeks until weaning approximately, mature OXT levels progressively rise (increasing blue font size from A. to E. represents this gradual rise over time). Both mature OXT and AVP are released by somas and developing dendrites in the offspring brain, while

axonal processes are still maturing at this time of age. **(E)**. By the fourth postnatal week, OXT and AVP neurons establish mature and long-range axons to multiple brain areas to release the neuropeptides, subsequently modulating specific brain function and behaviors. Figure adapted from *Grinevich et al. (2015)*²⁵⁵. Created with BioRender.

In parallel with the expansion of their receptors expression in the developing brain^{269,278,279}, OXT and AVP act on their own neurons through somato-dendritic release during the first two postnatal weeks (see **Figure 12C.**). This enables the depolarization of their own cells in the hypothalamus, facilitating their electrical activity and the increase in neuropeptide release^{275,276}. This positive feedback loop, called auto-control, is selective, as OXT and AVP activate the electrical activity of their neurons, although cross-activation (*e.g.*, through OXT binding on AVPR1a receptors, for instance) is not excluded²⁷⁵. The role of OXT and AVP auto-control initiates a succession of physiological events that contribute to the maturation of their neurons²⁷⁶, including membrane potential stabilization²⁷⁵, a significant dendritic outgrowth^{276,277} and the appearance of excitatory glutamatergic signaling²⁷⁷. More particularly, somato-dendritic release of OXT and AVP leads to the autocrine regulation of calcium stores mobilization which promotes both the maturation of local excitatory glutamatergic synapses and cell dendrite formation (see **Figure 12**)^{260,277}. Concomitantly, OXT contributes to the GABA switch event (*i.e.*, a shift from GABAergic excitatory to inhibitory neurotransmission)^{260,277}. Thereafter, membrane electrical properties and the dendritic tree organization are nearly fully shaped at the end of the second postnatal week²⁷⁶. Taken together, the first two postnatal weeks are paramount for the maturation of oxytocinergic and vasopressinergic processes within the developing rodent brain. However, the exact developmental timing of OXT axonal release in the brain is not fully elucidated yet, especially to distant areas such as the forebrain and the spinal cord. Therefore, it is proposed by *Muscatelli and colleagues*²⁶⁰ that somato-dendritic release is the main OXT release modality in the first weeks of life (see **Figure 12**), and that OXT might reach distant brain areas through a passive spread to the brain and/or in the CSF²⁸⁰.

Development and organization of the OXT and AVP systems projectome. Once established in adulthood, OXT and AVP axons send collaterals to numerous brain regions, which underlie their multiple roles in socio-emotional and reproductive behaviors, body metabolism, cognition, state control and pain, among other roles²⁶⁶. In the OXT system, OXT cells from the PVN rather innervate forebrain regions, whereas SON OXT cells send projections to limbic brain regions, the thalamus, hindbrain areas, and to the hypothalamus itself^{252,281}. Most brain areas innervated by OXT axon collaterals express the OXTR, including the hypothalamus, the lateral

septum, the auditory cortex, the central amygdala (medial and lateral subdivisions)^{282,283}, the hippocampus, the PFC, the NAc, among other brain areas (see **Figure 13**)^{266,267,281,284–286}, which all belong to the social and emotional brain network²⁸⁷.

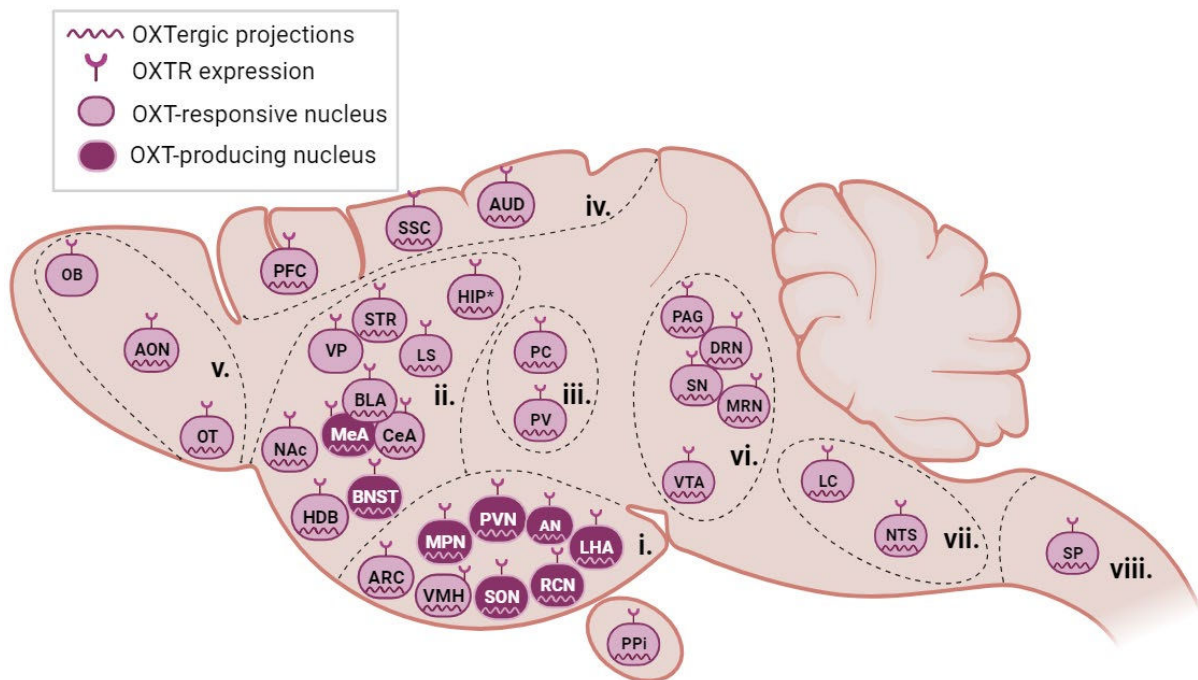


Figure 13. Localization of OXT neurons, projections and OXTR expression in the rodent brain.

This scheme summarizes the current knowledge on the brain oxytocin (OXT) system, including OXT neurons (OXT-producing nucleus), oxytocinergic projections (OXTergic projections) and OXTR expression (OXT-responsive nucleus) in the whole rodent brain. **(i.) Hypothalamus.** OXT-producing nucleus including the paraventricular nucleus (PVN), the supraoptic nucleus (SON), the accessory nucleus (AN), the retrochiasmatic nucleus (RCN), the lateral hypothalamic nucleus (LHA) and the medial preoptic nucleus (MPN). The most innervated output areas are found in the posterior pituitary (PPI), the PVN, the SON, the LHA, the MPN, but also in the arcuate nucleus (ARC) and the ventromedial hypothalamus (VMH) **(ii.) Cerebral nuclei.** Pre-limbic regions such as the striatum (STR), hippocampus (HIP, CA1 and CA2 subregions*), the lateral septum (LS), the amygdaloid complex (basolateral amygdala: BLA; medial amygdala: MeA; central amygdala: CeA), the nucleus accumbens (NAc), the horizontal limb of diagonal band (HDB). The BNST and the MeA are two extra-hypothalamic regions containing OXT neurons. **(iii.) Thalamus.** The periventricular thalamus (PV) and piriform cortex (PC). **(iv.) Cortical areas.** The prefrontal cortex (PFC, the prelimbic, infralimbic and anterior cingulate areas) and the neocortex such as the somatosensory (SSC) and auditory cortex (AUD) **(v.) Olfactory areas.** The anterior olfactory nucleus (AON) and the olfactory tubercles (OT). **(vi.) Midbrain.** Mesolimbic dopaminergic regions, including the ventral tegmental area (VTA), the periaqueductal gray nucleus (PAG), the substantia nigra (SN), the midbrain reticular nucleus (MRN) and the dorsal raphe nucleus (DRN). **(vii.) Hindbrain.** The locus coeruleus (LC) and the nucleus of the

solitary tract, receiving vagal afferents. **(viii.) Spinal cord (SP).** Of note, the existing literature did not demonstrate axonal projections to the olfactory bulbs (OB) and ventral pallidum (VP), although they are known OXTR-expression brain areas. Figure adapted from *Muscatelli et al. (2017)*²⁶⁰ and other references^{249,266,267,288,289}. Created with BioRender.

In addition, vasopressinergic outputs from the PVN are mostly located in the LHA, in mesolimbic dopaminergic regions (*e.g.*, NAc, ZI, MRN and the PAG), as well as cortical regions including the PFC²⁶⁷, among other brain regions. The densest AVP projections are found in the LHA, the NAc and the CPu, and thus AVP projection patterns are mostly concentrated around the hypothalamus and the thalamus in rodents²⁶⁷ (see **Figure 14**). In addition, the expression of AVP receptors (*i.e.*, AVPR1a and AVPR1b) is partly similar to the OXTR pattern. The receptor AVPR1a is the main vasopressin receptor expressed centrally^{290,291}, and is found in cortical regions (*i.e.*, piriform and somatosensory cortices)²⁷⁹, limbic brain regions (*i.e.*, CeA, LS, BNST, LHA, ARH, and the hippocampus)^{256,292,293}, as well as the spinal cord²⁹⁴. Of note, AVPR1b is also present in several central areas such as the hippocampal CA2 subregion, the PVN and the olfactory bulbs^{295–297}. AVPR1b is far less studied than its AVPR1a homolog but some studies show a potential role for mediating AVP roles in aggression and social behaviors across development^{214,215,297}.

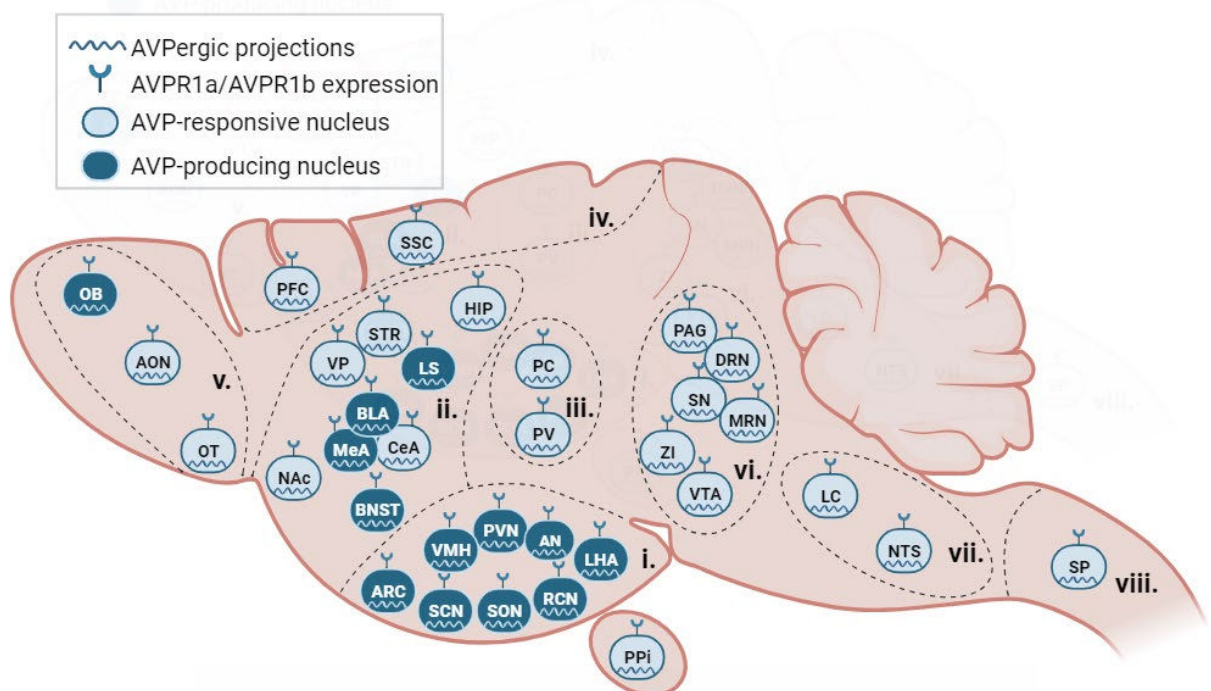


Figure 14. Localization of AVP neurons, projections and AVPR1a/AVPR1b expression in the rodent brain. This scheme summarizes the current knowledge on the brain AVP system, including AVP

neurons (AVP-producing nucleus), vasopressinergic projections (AVPergic projections) and AVPR1a/AVPR1b expression (AVP-responsive nucleus) in the whole rodent brain. **(i.) Hypothalamus.** AVP-producing nucleus including the paraventricular nucleus (PVN), the supraoptic nucleus (SON), the accessory nucleus (AN), the suprachiasmatic nucleus (SCN), the retrochiasmatic nucleus (RCN), the lateral hypothalamic nucleus (LHA), the ventromedial hypothalamus (VMH) and the arcuate nucleus (ARC) ^{267,294,298}. **(ii.) Cerebral nuclei.** Pre-limbic regions such as the striatum (STR), the ventral pallidum (VP), the hippocampus (HIP), the amygdaloid complex (basolateral amygdala: BLA; medial amygdala: MeA; central amygdala: CeA) and the nucleus accumbens (NAc). The bed nucleus of the stria terminalis (BNST), the BLA, the MeA and the lateral septum (LS) are extra-hypothalamic regions containing AVP neurons. **(iii.) Thalamus.** The periventricular thalamus (PV) and piriform cortex (PC). **(iv.) Cortical areas.** The prefrontal cortex (PFC) and the neocortex such as the somatosensory (SSC). **(v.) Olfactory areas.** The anterior olfactory nucleus (AON) and the olfactory tubercles (OT). **(vi.) Midbrain.** Mesolimbic dopaminergic regions, including the ventral tegmental area (VTA), the periaqueductal gray nucleus (PAG), the substantia nigra (SN), the midbrain reticular nucleus (MRN) and the dorsal raphe nucleus (DRN) and the zona incerta (ZI). **(vii.) Hindbrain.** The locus coeruleus (LC) and the nucleus of the solitary tract, receiving vagal afferents. **(viii.) Spinal cord (SP).** Figure adapted from *Grinevich and Ludwig (2021)* ²⁸⁸ and other references ^{267,279,282,290,291,293–297}. Created with BioRender.

Of importance is the sex bias in the shaping of the OXT and AVP systems. Indeed, it is well established that AVP levels in the hypothalamus are dependent on the testosterone levels in males, and the highest the testosterone content is, the lower AVP levels are ²⁹⁹. It was shown recently that, although the postnatal shaping of the OXT neurocircuitry is globally similar between sexes, the AVP input-output architecture is sexually biased ²⁶⁷. Indeed, the adult female brain send more AVP projections to the PFC and the NAc than males, whereas the adult male brain display a higher density of AVP projections to the ZI and midbrain areas (*e.g.*, the MRN and the PAG) compared to females ²⁶⁷. These shifts in the proportions of AVP projections density in the whole brain might underlie sex differences in the regulation of behavior.

Developmental trajectories of OXT and AVP receptors. Within these targeted brain regions, OXT and AVP signal through their respective receptors, the OXT receptor (OXTR) and the vasopressin receptors AVPR1a and AVPR1b ^{247,269}. Similar to the development of neuropeptidergic cell and axonal processes, the expression of OXTR and AVPR1a/AVPR1b is influenced either by age, sex, species, or a combination of these factors ^{269,278,286,290}. The mouse brain display a distinct spatiotemporal organization of OXTR expression ²⁶⁹, where OXTR expression is detected at E12.5 and is active from E16.5 in the ventricular and subventricular

zones and the developing amygdala²⁷³. Moreover, OXTR mRNA levels progressively increase in the neonatal mouse brain until adulthood, except for the neocortex in which OXTR expression peaks transiently at P14 and is eventually lateralized into the right cortex²⁸⁵. Likewise, OXTR expression shows a transient peak in the lateral septum (LS) and the hippocampal CA3 subregion during the whole third postnatal week²⁷⁸. In general, OXTR expression increases in an age-dependent manner in the olfactory bulbs, the BNST, the striatum, the piriform cortex, the CeA, MeA and the hypothalamus²⁷⁸, reaching an adult-like pattern by the time of weaning^{255,278}. In parallel, the OXTR expression is higher in regions regulating social behavior and spatial memory at the juvenile age whereas it is more highly expressed in cortical regions controlling social-decision making (*e.g.*, sexual partnership) in adulthood²⁴⁷. Of note, some OXTR-expressing areas do not receive axonal collaterals from the OXT neurons (*e.g.*, the olfactory bulbs), suggesting a possible volume diffusion and/or indirect effect of the neuropeptide in these specific areas^{249,260,285,300} (see **Figure 13**). Nonetheless, some studies are showing sparse OXT axonal projections to OXTR-expressing forebrain areas^{251,285}, suggesting that there may still be some undiscovered OXT fibers between the hypothalamus and the forebrain.

Similar to the OXT system, AVP binding sites are detected in the brain and spinal cord as early as the E16.5 stage, in significant levels in the SCH, the pons, the whole pituitary gland and the spinal cord⁵⁸. Postnatally, AVPR1a developmental pattern evolves progressively to reach an adult-like pattern around the time of weaning in mice and stabilizes in quantity and location into adulthood^{301,302}. At birth, AVPR1a peak of expression is detected in the neocortex, the septum, the hippocampal CA1 subregion and the ventral tegmental area (VTA)³⁰². In addition, AVPR1a expression pattern is transient in the neocortex (*i.e.*, the S1 cortex) and the hippocampal CA1 subregion in the first postnatal weeks of life, with a peak of expression at P7 in both male and female neonates³⁰². AVPR1a genetic loss in the mouse brain leads to sex-dependent defects in parvalbumin interneuron morphology and population numbers in the adult S1 cortex, while the transient peak of AVPR1a expression in the neocortex is also preserved in the human developing brain³⁰². These data support the functional relevance of AVPR1a expression in the neocortex for brain functioning later in development, and suggest that AVPR1a signaling pathway during the first postnatal weeks may have a role in the establishment of the excitatory/inhibitory cortical balance, and more generally in cortical information processing later in life³⁰². By contrast, AVPR1a expression pattern is relatively stable in the septal area and VTA across postnatal development of the mouse brain, with

potential female-specific variations in the peripubertal period in the VTA ³⁰². Of note, vasopressin receptors binding density increases significantly in an age-dependent manner in the piriform cortex and dentate gyrus in the developing rat ²⁷⁹, as well as in the NAc shell and the LS ²⁹⁰.

Importantly, neuropeptide receptors expression and cell projections are also sexually dimorphic. In the prenatal period, OXTR gene expression is significantly higher in females than males between E16.5 to P2 ²⁷³. Likewise, the OXTR is highly expressed in the PVN, the LS, the auditory cortex, the amygdala, the hippocampal CA2 subregion, the prelimbic division of the PFC, the olfactory bulbs and the raphe nucleus in virgins and pregnant adult females compared to males ²⁸⁵. By contrast, the adult male brain display lower intensity of OXTR expression in these brain regions, even lower than virgin females, however the density of OXTR in the hippocampus is more homogenous in the adult male brain than in the female. Moreover, sex hormones, including estrogens and testosterone, directly influence the OXT system and OXTR binding density is strictly diminished in adults rodents after gonadectomy ³⁰³, especially in brain areas controlling the reproductive functions (*e.g.*, the CeA, VMH, BNST and the LS, among others) ³⁰³. Likewise, AVPR1a binding pattern is also sexually dimorphic in the adult rodent, but in a smaller number of brain areas than OXTR patterns. Indeed, AVPR1a binding density in males *vs.* females is higher in the LS, the ARC and ventromedial thalamus, which belong to the social decision-making brain network ²⁹⁰. Moreover, sex differences in both OXTR and AVPR1a expression patterns are already visible at the juvenile age, suggesting that OXT and AVP are important in early life for sex-dependent social interactions.

Modulation of inhibitory and excitatory neurotransmission by OXT and AVP. OXT and AVP are mainly neuromodulators of the inhibitory neurotransmission. Indeed, OXT collaterals and/or OXTR expression are found in GABAergic interneurons of the LS ²⁸¹, the PFC ^{304,305} and the hippocampus ²⁴⁷, but also in GABAergic, parvalbumin- and somatostatin-positive inhibitory interneurons of the neocortex ²⁸⁴. Surprisingly, OXT/OXTR signaling modulates the synaptic inhibition in the in the PVN, the auditory cortex and the piriform cortex, therefore allowing an efficient processing of social information and adequate behavioral responses ^{284,285}. Similarly, the OXT system is involved in the regulation of the positive reward system in the mesolimbic VTA-NAc circuitry through the dopaminergic neurotransmission, either directly or indirectly by the projection to neurons of the prelimbic subregion of the PFC, the amygdala and the NAc ³⁰⁶. By contrast, OXT and glutamate co-release modulate the fear response in the CeA, with an alternating release of a slow-acting oxytocin and fast-acting release of the glutamate in

a fear-conditioning rat model^{252,300}. Taken together, these findings show that the OXT system modulate several neurotransmitter signaling pathways to perform a complex regulation of socio-emotional behaviors in rodents³⁰⁶. In fact, the complex modes of actions of OXT may be allowed by numerous release modalities to its neural targets. The OXTR is not only expressed in the ‘classical’ axonal ending button, but also along axons (in axonal varicosities), in cell bodies and dendrites³⁰⁶ and is expressed in non-neuronal neighboring cells, such as glial cells²⁸⁵, adding another complexity to the neuromodulatory effects of OXT. Interestingly, OXTR suppression leads to abnormal microglial morphology in the MeA and the LS, as well as a significant reduction in the post-synaptic PSD-95 associated to decreased ultrasonic communication in neonates at P5. Minocycline-induced inhibition of microglia between E15.5 and P21 rescues these neurobiological and behavioral deficits³⁰⁷, indicating that the OXT system would regulate synaptic development and maturation via microglial activation particularly in social-related brain region such as the MeA and the LS, which are involved in the modulation of early life communication. As microglia is believed to participate in synaptic pruning during development, it appears that the OXT system would be pivotal for the normal development of the social brain, and defects in OXT-microglia crosstalk would very likely lead to NDD such as ASD. Similarly, *Avpr1a* and *Avpr1b* are expressed in GABAergic interneurons of the CeA²⁹³ and in the hippocampal CA2 subregion²⁹⁶, respectively. A recent study²⁹³ also showed that AVPR1a-positive cells co-express the glutamate transporter VGLUT2 in synaptic terminals of the CeA of the female mouse brain²⁹³, thus suggesting that OXT and/or AVP might modulate the plasticity of glutamate synapses³⁰⁸. Altogether, this indicates that the vasopressinergic system modulate several neurotransmitter signaling pathways as the OXT system.

3.1.2 Regulation of socio-emotional behaviors by oxytocin and vasopressin

The complex neuroanatomy and release modalities of the OXT and AVP systems in the brain thus reflects their pleiotropic functions²⁴⁸. Their main functions are the regulation of social behaviors (*e.g.*, socio-sexual behaviors, maternal behaviors and attachment, general pro-sociality between conspecifics, social recognition), anxiety-like behavior, the fear/stress responses (notably by acting on the HPA axis, see next section), which rely on the association of multiple brain areas involved in sensorimotor functions, autonomic functions or cognition^{266,267} (see **Figure 15**).

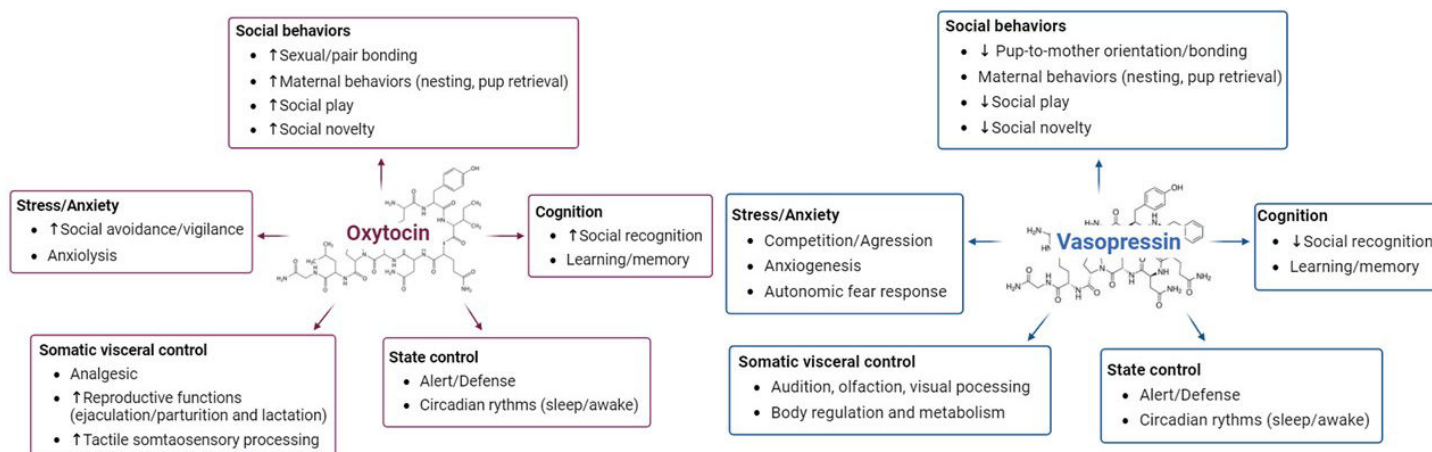


Figure 15. Behavioral and physiological functions of oxytocin and vasopressin in mammals. The neuropeptides OXT and AVP are involved in five main behavioral and physiological principal processes, naming cognition, state control, somatic visceral control, stress and anxiety responses, and social behaviors. Moreover, OXT and AVP may act in an opposite fashion *e.g.*, for the control of stress and anxiety behaviors. Figure adapted from *Patisaul (2017)*³⁰⁹ and other references^{269,288,310–312}.

Oxytocin, vasopressin, and the development of fear response and anxiety. It is now widely known that OXT and AVP exert opposite actions on fear and anxiety-like behaviors, as OXT is referred to as anxiolytic whereas AVP is anxiogenic^{269,310,312} (see **Figure 15**). Nonetheless, stressful and/or fearful stimuli trigger the release of both neuropeptides within the hypothalamus^{300,313}, probably through a somato-dendritic release modality³¹³.

Anxiety-like behavior and fear implicate numerous brain regions including the amygdala and the PFC. In the PFC, the OXT system activate the GABAergic interneurons in the prelimbic, but not the infralimbic and cingulate areas, of the PFC to reduce anxiety-like behavior in rats. Of note, this GABAergic-mediated anxiolytic action might be due to projections of the medial PFC GABAergic interneurons projections to the amygdala³¹⁴. Similarly, the fear response is attenuated by the oxytocinergic system, through the activation of GABAergic interneurons in the lateral CeA^{252,292}, themselves projecting to neurons of the medial CeA^{252,283}. In particular, inhibited neurons of the medial CeA project to the periaqueductal gray nucleus of the brainstem²⁸³, the autonomic hub of the fear response. Another mechanism by which OXT buffers fear is through the capacity of parvocellular and magnocellular cells to ‘imprint’ previously encountered aversive stimuli. In a non-fearful context, parvocellular OXT neurons projecting to the CeA mainly release OXT whereas a switch to glutamate neurotransmission occurs in a fearful context (probably because of the faster neurotransmission of glutamate compared to OXT and the necessity to ‘react’ rapidly to the potentially threatening situation). Secondly, a pool of parvocellular PVN OXT neurons project to magnocellular SON OXT neurons, which send

projections to both the CeA and the PPI. Interestingly, this pool of connected parvocellular and magnocellular OXT neurons are able to ‘record’ a fearful event, by differential activation and/or re-activation depending on the novelty of the fear stimulus³⁰⁰. Thus, the OXT signaling pathway in the CeA adapts to novel and familiar stressful conditions through OXT and glutamate co-release from SON magnocellular neurons concomitantly projecting to the CeA and to the periphery. Through this fine-tuned mechanism, the oxytocinergic neurotransmission in the CeA is thus plastic and allows a homeostatic adaptation to excessive stress after novel fearful experiences. Complementary to its anxiolytic/anti-fear role, OXT promotes hippocampal neurogenesis in adult rodents following stressful situations, very likely preventing the deleterious effects of glucocorticoids (*e.g.*, corticosterone) on neurogenesis³¹⁵. Overall, OXT is anxiolytic notably by slowing down (1) the CeA-PAG autonomic signaling pathway(s) to fear, (2) gating fearful stimuli depending on the context and allowing an adequate fear response, and (3) preserving cognitive functions in aversive conditions. In the case of chronic social defeat stress in females, OXT also facilitates social vigilance towards unknown conspecifics, probably preserving the stressed individual from another aversive encounter³¹⁶. In that context, OXT/OXTR signaling in the BNST of females, but not males, mediates this type of behavior, and projections from OXTR-expressing neurons of BNST to the NAc suggest a potential PVN-BNST-NAc pathway through which OXT favors social vigilance in females³¹⁶.

By contrast, the AVP system promotes the fear response, by the activation of differential and complementary neuronal circuitries in the CeA, the basolateral amygdala (BLA) and the amygdaloid subdivision of the BNST^{282,293}. By the projections of magnocellular AVP neurons of the HNS and the expression of the AVPR1a in GABAergic and glutamatergic neurons in these brain areas, the AVP system facilitates anxiety-like behavioral responses to physiological (*e.g.*, water deprivation) and psychological (*e.g.*, social defeat stress) stressful stimuli in rodents²⁹³. Importantly, AVP integrates excitatory synaptic information from the BLA projecting to AVPR1a-expressing neurons of the CeM, leading to direct activation of the autonomic fear response²⁹². By contrast, OXTR-responsive GABAergic cells of the CeL integrate information from cortical circuits and project to AVPR1a-expressing cells of the CeM. Through this latter projections, OXTR-expressing inhibitory interneurons can inhibit the autonomic fear response conveyed from AVPR1a-expressing neurons of the CeM to the brainstem²⁹². Through this mechanism, AVP and OXT interact to balance the autonomic fear response by acting on the transmission of information relayed through the brainstem. Moreover, it was shown that

suppression of AVP-positive cells in the BNST leads to sex-dependent effects on sickness behavior, characterized notably by increased anxiety-like behavior³¹⁷. Thus, AVP in the BNST might be relevant for the modulation of emotional and motor deficits observed in the context of sickness behavior in mice. In a stressful context, the presence of a conspecific buffers the stress-related synaptic plasticity controlling the stress response, and is called ‘social buffering’. Recently, *Lowen and colleagues*³⁰⁸ have demonstrated that social buffering between females, but not males, is mediated by the AVP/AVPR1a system in the PVN following a foot sock stress.

Oxytocin, vasopressin and social behaviors. It is now well established that OXT and AVP are involved in the regulation of social behaviors in rodents (see **Figure 15**). In male mice, social interactions with conspecifics activate PVN OXT neurons, which in turn promotes social behavior, thus showing a virtuous cycle between OXT neurons and sociability³¹⁸, where the OXT system encodes positive social stimuli and orientates the individual to engage in social behavior. Supporting this view, the Shank3B-deficient mouse model of ASD display less PVN OXT neurons, thus resulting in decreased social behavior compared to typically developed mice. Interestingly, an OXTR receptor agonist reduces the number of OXT neurons and ameliorates the social deficits characteristic of Shank3B-deficient mice³¹⁸. A recent pivotal study²⁵⁴ shows that physical social interactions in adult female rats potentiates the firing activity of OXT neurons in interfemale social play behavior. Mechanistically, dorsal physical contacts activate the parvocellular population of PVN OXT neurons that fire to magnocellular neurons, leading to positive social behavior in the female rat²⁵⁴. These OXT magnocellular neurons of the PVN release OXT in the periphery while promoting social interactions centrally, suggesting a potential dual regulation of sociability in rodents^{251,254}. Furthermore, the OXT system is required for social recognition, and is mediated by the expression of OXTR on GABAergic interneurons and glutamatergic pyramidal neurons of the PFC, sending projections through both GABA and glutamate in the NAc, BNST and BLA³¹⁹. Although spatiotemporal activation of either neurotransmitter signaling is not understood yet for the regulation of social recognition, it is possible that the oxytocinergic system fine-tune both GABAergic and glutamatergic signaling depending on the environmental stimulus. In the BNST, AVP cells particularly regulate social recognition in a sex- and context-dependent manner, as the absence of AVP cells in this precise brain region attenuates male social recognition behavior³¹⁷.

Prosocial effects of OXT might also be mediated by its action on the mesolimbic dopaminergic reward system. Through its axonal projections and the OXTR expression in the midbrain dopaminergic regions, the OXT system, and probably AVP, exert a two-fold modulation over

the dopaminergic reward system³²⁰, by increasing the activity of the VTA dopaminergic neurons and indirectly decreasing the dopaminergic neuronal activity of the *substantia nigra pars compacta* (SNc) through the GABAergic signaling. In the VTA, OXT can signal through the OXTR and the AVPR1a, which are expressed by dopaminergic and GABAergic neurons. In that region, OXT-responsive GABAergic neurons inhibit the VTA dopaminergic neurons, providing a ‘brake’ over dopaminergic neuronal excitability. By contrast, the same parvocellular OXT neurons project to OXT-responsive GABAergic neurons of the SNc inhibiting dopaminergic neurons of the same brain regions, thus only acting as an indirect inhibitory neuromodulator in that region³²⁰. Recently, *Lewis and coworkers*²⁵⁶ showed that parvocellular OXT neurons innervating the NAc requires the autism-risk gene *Fmr1* (known to be mutated in Fragile X syndrome, a genetic form of autism) to mediate social reward behavior towards conspecifics in mice. Of note, autism-risk genes such as *Fmr1* are enriched specifically in parvocellular OXT neurons compared to magnocellular neurons²⁵⁶. From these observations, one may hypothesize that parvocellular OXT neurons targeting dopaminergic neurons controlling social behavior, may have a specific role in the pathogenesis of ASD. An important study from *Hörnberg and colleagues*³²¹ also demonstrates that the oxytocinergic, but not the vasopressinergic, signaling in VTA dopaminergic neurons promoting social novelty behavior depends on the synaptic adhesion molecule Nlgn3. In particular, Nlgn3 gene deficiency in mice leads to social novelty deficits, that are already visible from the juvenile period³²¹. Also, this protein regulates the mRNA translation rate through its action on a phosphorylation protein that controls the initiation of translation. Pharmacological inhibition of the phosphorylation of proteins involved in mRNA translation rescues the cell response to OXT, neuronal firing activity and social deficits observed in Nlgn3-deficient mice³²¹. Furthermore, Nlgn3 might be required for the recruitment of the OXTR to the cell membrane, as loss of Nlgn3 is associated to alterations in proteins involved in protein transport, cell adhesion, GPCR signaling and membrane trafficking³²¹. Taken together, this study reports a significant link between OXT signaling, cell signaling, mRNA translation and the development of social behavior, and supports a significant molecular target for the treatment of NDD.

Importantly, social interest is positively correlated to OXTR density in males, but negatively in females, suggesting that the OXT-mediated social behavior is established differently between males and females. Also, higher OXTR expression, especially in the MeA, may facilitate male pro-social behavior²⁸⁶. Furthermore, socio-sexual behaviors is also regulated differently between sexes. Female socio-sexual behavior, particularly during the estrus phase (*i.e.*,

ovulation period in rodents during which females are the most sexually receptive), is regulated through a specific population of somatostatin-expressing interneurons via the OXT signaling pathway in the mPFC³⁰⁴. More precisely, it was shown that oxytocin triggers neuronal firing in a population of somatostatin interneurons and some GABAergic cells of the mPFC, more markedly in females compared to males. At the behavioral level, inhibiting the OXTR signaling pathway in female interneurons leads to decreased social preference towards a male over an inanimate object, revealing the potential role of OXTR-expressing interneurons in social interest for a sexual partner in females³⁰⁴. The fact that this socio-sexual behavior is specifically regulated by OXT during the estrus phase might be relevant since there is an increase in peripheral OXT at this time of the cycle³⁰⁴. Also, there is likely a cross-system signaling between OXT and AVP, specifically for the regulation of social behaviors, and some studies revealed that it would be age-, sex- and context-dependent²⁹¹. For instance, in the mouse brain, OXT can regulate sociability and social recognition in OXTR-deficient mice thanks to its binding to AVPR1a, showing clearly an OXT/AVPR1a cross-signaling pathway in rodents for the control of social behavior³²², that may occur as a compensation when the initial cognate receptor is suppressed. Moreover, spatial differences in OXTR and AVPR1a expression patterns, as well as their respective projection patterns, suggest a potential cross-signaling between the OXT/OXTR and the AVP/AVPR1a systems²⁹¹.

During early life, the OXT system already plays a critical role in preparing the brain to diverse social stimuli. Several studies^{279,280,318} have shown that negative and positive social-related experiences shape the maturation of the OXT system. For instance, early life stress (*e.g.*, maternal separation) is detrimental for the proper development of OXTR or AVPR1a binding in the mouse brain²⁷⁹. Likewise, early deprivation of sensory experiences (*e.g.*, whisker deprivation or limited nesting material in the first weeks of life) reduce the OXT cell activity in the PVN²⁸⁰, as well as altered OXTR expression levels in the PVN, CeA and hippocampal CA3 subregion at P15³²³ and sex-dependent changes in the numbers of OXT cells in the PVN and OXT-responsive neurons in the CeA and BLA³²³. These OXT neurochemical changes has important implications for related social behaviors later in life since deprivation of nesting material in the first two weeks of life results in lower social interest in females, but not males³²³. These important findings show that early life sensory experiences, related to the first social experiences (*e.g.*, maternal and littermates contacts) are paramount for the proper establishment of the OXT system in the brain. This has important implications later in life, although social experiences also modulate the OXT system towards social engagement even in adulthood.

Indeed, positive social stimuli in adult male rats recruit and activate PVN OXT cells, promoting social behavior later in the adult male brain and favoring cell plasticity in the PVN ³¹⁸.

3.1.3 Development of the CRH system and the HPA axis

The CRH system in the HPA axis. Following stress exposure, the hypothalamus is stimulated so there is a two-step stress response, a quick neurotransmitter-mediated signaling pathway and a slower neuroendocrine response ³²⁴. In the quick stress response, mediated by the sympatho-adrenal medullary axis, the adrenal medulla produces catecholamines (*e.g.*, adrenaline and noradrenaline), that rapidly engage physiological processes typical of the ‘fight-or-flight’ response, including increasing heart rate, blood pressure or gluconeogenesis ³²⁴. The long-term neuroendocrine response is allowed by the HPA axis, the release of the corticotropin-releasing hormone (CRH), a neuropeptide produced by cells of the hypothalamic PVN ³²⁵. In turn, CRH activates corticotrope cells of the anterior pituitary, which triggers the release of the ACTH into the bloodstream and periphery. Next, ACTH stimulates the production of glucocorticoids (*e.g.*, corticosterone in rodents and cortisol in humans) and mineralocorticoids from the adrenal cortex ^{324,326} (see **Figure 4**). In particular, glucocorticoids activate several central and peripheral areas including ones involved in lipid and protein catabolism, in the activation of the ANS, in the inhibition of reproductive and immune functions, and in enhanced cognition, reinforcing the ‘fight-or-flight’ response ^{324,326}. Importantly, the HPA axis is a fine-tuned system involving a negative feedback loop with the action of peripheral glucocorticoids on glucocorticoid and mineralocorticoid receptors (respectively, GRs and MRs), in the hypothalamus, the anterior pituitary gland and the adrenals, but also in related brain regions such as the hippocampus and the PFC ³²⁶. In the hypothalamus, the neuropeptide CRH is abundantly produced in parvocellular neurons of the lateral-most divisions of PVN ³²⁵, where CRH neurons are mostly glutamatergic (*i.e.*, VGLUT2 expression), and to a lesser extent, GABAergic ³²⁷. Importantly, the neuropeptide is also expressed in extra-hypothalamic regions including the CeA ³²⁸, the BNST ³²⁷, the hippocampus and several cortical brain regions ³²⁵. In the anterior BNST and in the peri-PVN, CRH neurons are solely GABAergic ³²⁷. The fact that CRH neurons in the PVN and BNST have distinct neurochemical and electrophysiological phenotypes, respectively a faster *vs.* a slower neuronal activation ³²⁷, suggest a differential modulatory effect over the HPA axis stress response. Following stress, several limbic structures, such as the BNST, send direct projections to PVN CRH neurons in a GABAergic and sex hormones-dependent manner. Moreover, the hippocampus, PFC, CeA, MeA and LS also send indirect inputs, either through the BNST or the peri-PVN, to modulate the activity of PVN CRH neurons in stressful contexts

³²⁵. Then, CRH neurons project to autonomic areas such as the brainstem and spinal cord ³²⁹, but also to other neuropeptidergic cells projecting to the median eminence, such as the magnocellular OXT and AVP neurons (see next subsection).

Moreover, CRH actions are mediated by its cognate receptors, the G protein-coupled receptors CRHR1 and CRHR2, that are differentially expressed across the brain ³²⁶. Although both are expressed in the hypothalamus and anterior pituitary gland, CRHR1 is found abundantly in cortical regions, the cerebellum and along the spinal cord, and is spread across limbic brain regions such as the striatum, the CA1 hippocampal subregion, the amygdala, the VTA, the NAc, among other regions ^{330,331}. By contrast, CRHR2 expression is more restricted, particularly to limbic brain regions such as the BNST and hypothalamus ³²⁷, as well as the LS, the MeA and the ventromedial hypothalamus ³³⁰ (see **Figure 16** below).

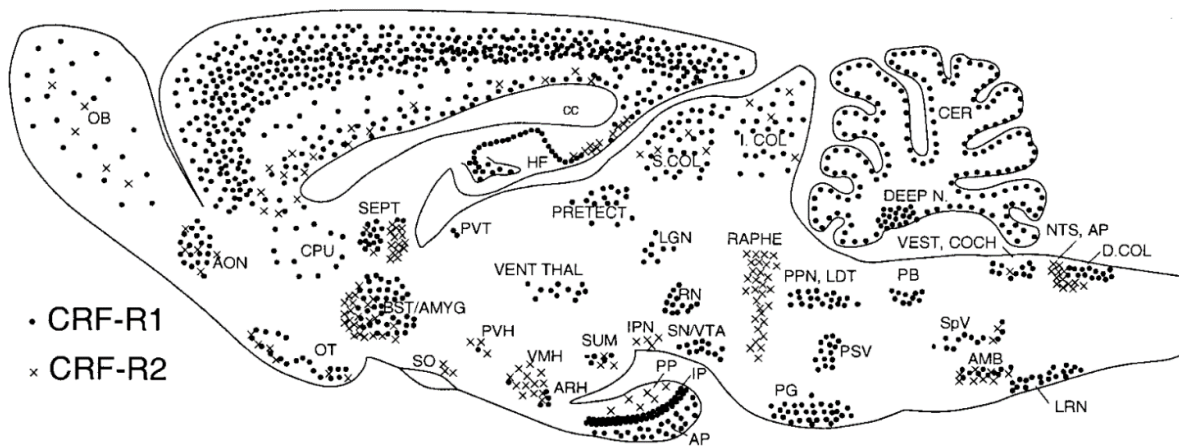


Figure 16. Expression of the CRH receptors, CRHR1 and CRHR2, in the rodent brain. This scheme represents the distribution and relative proportions of CRHR1- and CRHR2-expressing cells, with CRHR2 expression being more restricted spatially than CRHR1. Abbreviations: OB olfactory bulbs, AON anterior olfactory nucleus, CPu caudate-putamen, SEPT septal region, OT, olfactory tubercle, BST/AMYG bed nucleus of stria terminalis/amygdala, SO supraoptic nucleus, PVH paraventricular nucleus, VENT THAL ventral thalamus, PRETRECT pretecal region, PVT paraventricular thalamus, S.COL superior colliculus, LGN lateral geniculate nucleus, SUM supramammillary nucleus, PP posterior pituitary, IP interposed nucleus, AP anterior pituitary, SN/VTA substantia nigra/ventral tegmental area, RAPHE raphe nucleus, PPN pedunculopontine nucleus, LDT laterodorsal tegmental nucleus, PSV principal sensory nucleus of the trigeminal nerve, PG pontine gray, DEEP N. deep nuclei of the cerebellum, CER cerebellum, VEST. COCH vestibular cochlear nuclei, AMB nucleus ambiguus, NTS nucleus of the solitary tract, D. COL dorsal column nuclei, LRN lateral reticular nucleus. Figure from *Van Pett et al. (2000)* ³³⁰.

Crhr1 expression is induced either by physiological or psychological stresses in the parvocellular division of the PVN, as well as in the magnocellular cells of the PVN and SON^{330,332,333}. Importantly, the CRH/CRHR1 system modulates the HPA axis stress response differentially in the brain, as it co-localizes with several neurotransmitter signaling pathways throughout the brain. In particular, *Crhr1* is densely and specifically expressed in cortical pyramidal neurons (likely glutamatergic) of the cortical layer IV, I and V, and GABAergic neurons^{331,333}. In the midbrain, *Crhr1* is expressed in dopaminergic neurons of the *substantia nigra* and VTA, whereas some GABAergic neurons expressing *Crhr1* are present in the reticular thalamic nucleus, global pallidus and septum³³³. Serotonergic neurons of the midbrain also express *Crhr1*³³³.

In addition, sex hormones are crucial regulators of the CRH/HPA axis, thus leading to strong differences in stress responsiveness between males and females³²⁶. In fact, high testosterone levels in castrated males inhibits the ACTH and corticosterone responses, but not CRH levels, in the median eminence, and increases the negative feedback loop (e.g., increased expression of the GR) in the medial preoptic area of the hypothalamus²⁹⁹. Similarly, estrogens modulate the HPA axis responsiveness, notably through the estrogens receptors ER α and ER β expression in core regions of the stress axis³²⁶. Progesterone also attenuates ACTH and corticosterone responses to emotional distress in rats³³⁴, showing the potential inhibitory effect of estrogens on the HPA axis responsiveness.

Development of the HPA axis and *Crh* expression. The development of the HPA axis starts in the prenatal life^{326,335}. In particular, *Crh* mRNA is detected at E17 in the PVN of the rat fetus and its levels are increasing until a few days before birth and then decreases until birth. From birth until the end of the first postnatal week, *Crh* gene expression increases progressively in the PVN³³⁵. As a consequence, the fetus possesses a rather functional HPA axis with the secretion of CRH and ACTH in response to maternal stress³³⁶. Nevertheless, the postnatal development of the HPA stress system is divided into two phases: a first phase, called the stress hypo-responsive period, which is characterized by high *Crh* mRNA levels in the PVN whereas basal ACTH and corticosterone levels are relatively low between P4 and P12³³⁷. Then, the second phase of the HPA axis development consists in increasing ACTH and corticosterone whereas *Crh* gene expression are decreased significantly, at least until P16. In parallel, a peak of GR expression is observed in the hippocampus at P12, which corresponds to the end of the stress hypo-responsive period in the neonatal mouse³³⁷. Thus, these developmental dynamics might be a strategy to protect the underdeveloped neonate towards excessive environmental

stressful stimuli in early life and to prevent HPA axis hyperresponsiveness. As a consequence, several environmental stressors, originating from the mother during the prenatal period, can interfere with the proper development and function of the HPA axis later in life. For instance, prenatal maternal stress was shown to upregulate *Crh* expression and provokes an excitatory/inhibitory imbalance in CRH neurons of the PVN of juvenile offspring later in life, with a hyperactive glutamatergic signaling in CRH neurons and the reduction of glutamate recycling by neighboring astrocytes³³⁶. This shows that the HPA axis development is vulnerable to abnormal excessive stress in the prenatal window leading to its hyperresponsiveness later in life. Later at the pubertal period, increasing levels of sex hormones interact with the HPA axis function, thus becoming sex-dependent³²⁸. After puberty, female, but not male, rats show higher plasma corticosterone levels compared to the prepubertal period under basal and stressful conditions³²⁸, confirming the strong sex differences in HPA axis reactivity in adult life. Similarly, basal *Crh* gene expression in the PVN increases with age in females whereas this age-dependent increase in *Crh* mRNA levels is observed only in the CeA in males³²⁸, indicating that the sex hormonal influence over HPA axis function might be established according to distinct neuronal networks between sexes.

Implications of the CRH/HPA axis in stress and anxiety. Interestingly, targeted deletion of *Crhr1* in limbic brain structures (*i.e.*, cortical regions, hippocampus, amygdaloid complex) results in reduced anxiety-like behavior, with a maintained peripheral stress response after restraint stress compared to full *Crhr1*-deficient animals, suggesting that the function of the CRH/CRHR1 pathway in the limbic brain contributes to stress-induced anxiety levels. Nonetheless, *Crhr1* suppression specifically in the limbic brain leads to stress hypersensitivity, notably with the impaired recovery of ACTH and corticosterone levels to basal levels over time³³². Furthermore, the limbic CRH/CRHR1 system interacts with *Mr* expression in the hippocampus, suggesting a potential role of the CRH/CRHR1 system in the MR-mediated negative feedback loop of the HPA axis³³². Thus, the CRH/CRHR1 seems to participate to the affective response to stress through the central regulation of the HPA axis. Likewise, there is evidence that *Crhr1*-expressing glutamatergic neurons in the cortex and hippocampus and *Crhr1*-expressing dopaminergic neurons of the *substantia nigra* and the VTA are respectively anxiogenic and anxiolytic, which provides a bidirectional and balanced regulation of the anxiety response in rodents in stressful contexts³³³.

The link between the OXT/AVP and the CRH systems. Both OXT and AVP are released in stressful contexts²⁶⁰ and stimulate ACTH secretion through AVPR1b in the corticotrope cells

of the anterior pituitary ³³⁸, while being released in the periphery after a stress stimulus. Of interest, *Crh* is co-expressed with *Oxt* or *Avp* in magnocellular and parvocellular populations of the PVN, respectively. CRH neurons also sparsely co-localizes with OXT in magnocellular SON neurons ³³⁹. For instance, parvocellular CRH neurons co-localize with *Oxt* and *Avpr1b* while BNST CRH neurons express *Oxtr* and *Avpr1b* receptors ³²⁷, suggesting that OXT anxiolytic effect over the stress response might be mediated by BNST CRH neurons, whereas PVN CRH neurons might be the source of OXT release to modulate BNST CRH neurons.

As mentioned above, OXT is anxiolytic, and this effect is largely mediated by its inhibitory action over the HPA axis function within the PVN, but not in pituitary cells ³⁴⁰⁻³⁴². Following stress, the OXT/OXTR system represses *Crh* gene expression ³⁴⁰⁻³⁴², by blocking the intracellular recruitment of one of its transcription factors, further delaying *Crh* gene transcription in the PVN and subsequent ACTH and corticosterone release in the periphery ³⁴³. OXT also modulates the HPA axis response in other brain structures, in a region- and context-dependent manner ^{340,342}. At basal levels, OXT inhibits the stress response, dampening peripheral ACTH release ³⁴⁰⁻³⁴², whereas it stimulates the ACTH response after a forced swim stress in the PVN of rats ³⁴⁰. By contrast, the elevated plate stress is dampened by the OXT/OXTR pathways in the LS and the amygdala ³⁴², but not after a forced swim stress or in basal conditions ³⁴⁰. The OXT system thus regulates the HPA axis response differently, depending on the type of stressor and by recruiting distinct neuronal networks. Similarly, OXT might hinder the HPA axis reactivity to stress through the activation of the HPA axis negative feedback loop. Indeed, OXT stimulates GR and/or MR-expressing hippocampal cells in psychologically-stressed animals and reduces *Gr* expression while increasing *Mr* expression ³⁴⁴. Even more so, OXT may facilitate an effective HPA axis response by increasing corticosterone levels immediately after stress exposure, whereas it represses the HPA axis reactivity several minutes after the stress exposure, by its action on the HPA negative feedback loop ³⁴⁴. Importantly, corticosterone not only contributes to the negative feedback loop through GR binding, but also by the activation of intrahypothalamic OXT secretion, by activating OXT release directly from OXT neurons and/or acting on CRH neurons stimulating PVN OXT neuronal activity in the PVN. Corticosterone might also be essential to regulate peripheral OXT levels since it activates GABAergic interneurons inhibiting PVN neurons, thereby controlling peripheral OXT release following stress ³⁴⁵. Corticosterone also synergizes with noradrenaline and upregulate hippocampal *Oxtr* expression ³⁴⁴, thus reinforcing the inhibition of HPA axis reactivity to stress. Interestingly, early life somatosensory stimuli are essential for the

development of OXT-responsive brain regions involved in the HPA axis regulation, notably deprivation of nest material in early postnatal life leads to increased *Oxt*- and *Oxtr*-expressing cells at P15 in the PVN, dorsal hippocampal CA3 subregion and the CeA, and sex-dependent changes in OXT cell numbers in the PVN in adulthood³²³. Females, but not males, have higher OXT cells in the PVN and *Oxtr*-expressing neurons in the PVN and CeA, which compensate for the HPA axis hyperreactivity induced by early life sensory deprivation. As a consequence, females might be more resilient to early sensory deprivation than males by overdeveloping OXT neuronal networks in the brain.

AVP is considered as part of the HPA axis and *Avp* is co-expressed in some CRH neurons of the PVN in humans³⁴⁶. Moreover, AVP neurons of the PVN are stimulated by corticoids in the HPA axis negative feedback loop³²⁶. In fact, testosterone infused in the medial preoptic area of the hypothalamus in male rats inhibits the production of AVP in the median eminence and downstream HPA responses, suggesting that sex hormones effects on the HPA axis are mediated, at least in part, through AVP in males. In females, injected progesterone reduces *Avp* mRNA levels in the PVN³³⁴, also indicating an effect of estrogens on AVP-mediated HPA response. It is also recognized that estrogens interact with OXT and AVP in the regulation of the HPA stress axis, as ER β neuroendocrine cells co-expressed *Oxt* and *Avp* in the PVN, and moderately co-localize with CRH cells in both male and female mice³⁴⁷. The sex-dependent effects of neuropeptides on HPA axis regulation even goes beyond the hypothalamus. In a pivotal study, *Li and colleagues*³⁰⁵ demonstrated that *Oxtr*-expressing GABAergic interneurons of the medial PFC modulate socio-emotional behaviors through two distinct pathways between male and female mice. Besides inhibitory GABA neurotransmission, these GABAergic interneurons have a repressing effect over anxiogenic *Crhr1*-expressing neurons of the cortical layers II/III, with the production of the inhibitory CRH binding protein. In parallel, the same interneurons inhibit the pro-social effect of layer V pyramidal neurons. In females, the OXT-responsive GABAergic interneurons leads to the repression of *Crhr1*-expressing neurons, but high CRH levels favors the direct activation of *Crhr1*-expressing pyramidal cells, and blunts the repressive action of OXT over these anxiogenic neurons, promoting anxiety-like behavior over positive social interactions. By contrast to females, low CRH levels in males promote the activation of OXT-responsive interneurons over *Crhr1*-expressing cells, leading to reduced anxiety-like behavior. In parallel, OXT inhibitory effect over layer V pyramidal cells is less potent in males, facilitating social behavior³⁰⁵. This study provides a clear evidence of sex-

specific interaction of the OXT and the CRH/CRHR1 system to modulate socio-emotional responses under basal and stressful conditions.

3.2 Gut microbes and neuropeptidergic systems

In the past decade, a large body of evidence points out to the implication of the gut microbiota on the modulation of OXT, AVP and CRH neuropeptidergic systems regulating socio-emotional behaviors.

3.2.1 Gut microbes in the development of the OT/AVP systems

There is increasing evidence of the influence of gut microbes on the development and function of the OXT and the AVP systems in the brain. In GF mice, the hypothalamic *Avp* gene expression is reduced, while *Oxt*, *Oxtr* and *Avpr1a* mRNA levels are unchanged in the hypothalamus³⁴⁸. By contrast, GF male rats, but not females, display a significant increase in OXTR binding activity in the prelimbic and cingulate PFC areas, the orbital cortex, the septum, and the NAc, and these effects are already visible from the end of the first postnatal week³⁴⁹, overall suggesting that colonizing gut microbes shapes the *Oxtr* gene expression patterns in a sex-, age- and region-dependent manner.

Interestingly, OXT supplementation in the first days of life improves social and anxiety-like behavioral defects of c-section-delivered pups, with a high OXT dose restoring behavioral defects both in early life and adulthood¹⁶⁶. As c-section delivery alters microbial colonization processes from birth onwards¹⁶⁶, these observations suggest that colonizing microbes might interact with neonatal OXT for the proper development of socio-emotional behaviors, although molecular mechanisms underlying this crosstalk has to be further investigated. The laboratory of M. Costa-Mattioli began to unravel the mechanisms underlying the influence of gut microbes on the developmental trajectories of the OXT system. Firstly, they showed that maternal high-fat diet-induced dysbiosis in offspring is associated to decreased OXT cell numbers, a reduced plasticity in the VTA and deficits in sociability and social recognition. Importantly, intranasal OXT administration and/or exposure to the commensal strain, *Lactobacillus reuteri*, reverses VTA plasticity impairments as well as behavioral deficits, indicating that *L. reuteri*, might potentiate the interaction of the OXT system and the dopaminergic reward system in social brain circuits¹⁸⁷. At the mechanistic level, they found that *L.reuteri* signals to the brain via the vagus nerve, itself projecting to dopaminergic midbrain areas such as the VTA. Moreover, the molecular crosstalk between the oxytocinergic and the VTA dopaminergic reward systems are required for the beneficial effect of *L.reuteri* on social behavior in genetic, environmental and

idiopathic autism mouse models⁸¹. Similarly, it was found that exposure to the probiotic strain *L. rhamnosus JB-1* restores *Avpr1a* and *Avpr1b* gene expression in the frontal cortex, hypothalamus and hippocampus, and corrects social deficits induced by low-dose penicillin administered during the postnatal period²¹⁵.

Similarly, daily administration with *Lactobacilli* strains, such as *L. helveticus* CCFM1076, between weaning and puberty rescues autism-related socio-emotional deficits, by normalizing the intestinal and central 5-HT neurotransmission (notably in the PFC) in the autism valproic acid-treated mouse model. *L. helveticus* CCFM1076 restores the balance in glutamatergic excitatory/GABAergic inhibitory signaling, as well as increasing hypothalamic OXT synthesis³⁵⁰. Thus, *Lactobacilli*, in particular the strain *L. helveticus* CCFM1076, may contribute to the development and function of 5-HT neurotransmitter signaling for the regulation of the OXT neuroendocrine system. As for the AVP system, *Avp* WT, heterozygous and KO genotypes in rats are associated with differential *Lactobacillus* abundance in their gut microbiota, with more elevated levels of *Lactobacilli* in *Avp* KO animals, more markedly in males than genotype-matched females³⁵¹. Interestingly, *Avp* KO animals show reduced anxiety³⁵² and increased levels of *Lactobacilli*³⁵¹, which reinforces the idea that these bacteria interact with neuropeptidergic systems for the regulation of socio-emotional behaviors. These findings confirm the importance of gut commensals, especially the *Lactobacillus* genus, to control the development and function of the OXT and AVP systems, through potential interactions with the vagal-dopaminergic and/or the serotonergic pathways. Furthermore, *Gur and colleagues*¹⁸¹ have shown that the interaction of OXT and the HPA axis functioning might be modulated by gut microbes in the prenatal period. In a prenatal stress mouse model, gut microbiota alterations are associated to social deficits, the overactivation of the HPA axis, and dysregulated serotonergic and OXT systems in the cortex¹⁸¹. This suggests that the coordinated function of the OXT system and the HPA axis to regulate the socio-emotional response to stress might be mediated through gut microbes in early life, although there is a large body evidence that the gut microbiota influences the HPA stress responsiveness through the CRH neuropeptidergic system.

3.2.2 Gut microbes in the development of CRH and the HPA axis

As mentioned before, the work from *Sudo and colleagues* (2004)⁶² is the first landmark study reporting the role of the gut microbiota in the development of the HPA stress axis in mice. These authors and others showed that the absence of microbes in the GF rodent model leads to an exaggerated HPA axis stress response following acute stress^{62,64,90}, which was also reported after a chronic stress exposure^{86,88}. GF mice show decreased anxiety-like behavior compared

to conventionally-colonized mice following stress exposure^{62,64,86,90,236,353}. By contrast to mice, the absence of microbiota in the stress-prone F344 rat strain induces increased anxiety-like behavior after an acute stressor⁸⁸. At the molecular level, GF^{62,64,86} mice show higher *Crh* mRNA and protein expression in the hypothalamus at basal levels, as well as increased hypothalamic *Crhr1* gene expression before and after chronic stress⁸⁶. Of note, acute restraint stress in the GF stress-prone BALB/c mouse leads to alterations in *Crhr1* gene expression in the pituitary⁸⁵. Furthermore, the absence of microbes in a stressful context leads to the hyperactivation of CRH neurons in brain regions relevant to socio-emotional behaviors, such as the PVN^{62,90}, the BNST) and the DG, which decreases social behavior⁹⁰.

Social interest is another behavioral component regulated by the gut microbiota in stressful contexts. Of note, this regulation is species-dependent, since social deficits are more marked in GF mice^{354,355} compared to GF rats. Indeed, GF stressed rats tend to engage less into social interactions compared to SPF rats, although they are overall as social as SPF counterparts⁸⁸. Fecal microbiota colonization rescues social deficits and hyperactivation of the HPA axis, demonstrating the pro-social role of the gut microbiota. One promising candidate in this beneficial regulation would be *Enterococcus faecalis*, as this bacterial strain facilitates sociability and dampens the social stress response³⁵⁵. Concurrently, the gut microbiota seems to participate in regulating defensive behaviors. After a social defeat stress experience, the ‘fight-or-flight’ response of GF mice is higher than conventionally-colonized control mice⁸⁷, suggesting a dampening role of the gut microbiota over defensive behaviors. Moreover, gene expression of the GR is lower in the cortex of GF mice compared to SPF mice after stress^{62,64,86}. Interestingly, repeated social stress upregulate the pituitary *Fkbp5* gene expression. This protein is involved in the regulation of GR sensitivity to corticoids, that is aggravated by the GF status, suggesting that commensal microbes potentially attenuate the negative feedback loop of the HPA axis by both acting on *Gr* gene expression and its affinity to corticoids⁸⁷. There might be a differential role of gut microbes on the HPA axis activation depending on the brain region involved. Taken together, these observations point out to a significant role of the gut microbiota to modulate the *Crh*/HPA axis in specific brain regions to regulate the stress levels and stress-related socio-emotional responses.

The time window during which the host is exposed to gut microbes is also crucial for the development of the HPA stress axis, since it was shown that reconstitution of the gut microbiota in GF mice during a specific window of early development restores the overactivation of the HPA axis, including the relatively high corticosterone plasma levels^{62,90}. Interestingly, stressful

stimuli during the prenatal period disturbs the normal development of the HPA axis, which results in exaggerated stress reactivity later in adulthood, accompanied with major physiological changes in the respiratory, circulatory and GI systems ¹⁷⁹. This is in accordance with a previous study ⁸⁸ indicating that early life stress insults is determinant for the central development of stress regulation and sensitivity later in life. Altogether, this suggests that the establishment of the HPA stress axis, particularly at the level of the neuropeptidergic CRH system, requires a normal gut microbial colonization during the early postnatal period. Some studies identified potential commensal strains ^{89,90} or microbial components ³⁵⁶ that mediate the impact of the gut microbiota on the HPA axis function. For instance, *L.rhamnosus JB-1* requires a crosstalk between regulatory T cells and vagal afferents to dampen stress-induced microglial activation in the hippocampus and anxiety-like behavior in stressed mice ⁸⁹. In another study ⁹⁰, exposure to *Enterococcus faecalis* in antibiotic-depleted mice rescues social stress-induced HPA axis hyperactivation, as well as sociability defects, altogether suggesting that *L.rhamnosus JB-1* and *Enterococcus faecalis* would be implicated in the modulation of the HPA axis response to stress.

If pre-clinical studies provide strong evidence that gut microbes are required for the regulation of the stress response, translation into humans has to be further investigated. By contrast to the findings of *Sudo and colleagues* ⁶², microbial diversity in human infants is positively associated to overactivation of the developing HPA axis stress response at one month of age ¹⁸⁶. In this clinical pilot study ¹⁸⁶, neonates with a more diverse microbiome (*e.g.*, increased α -diversity) display a higher stress response (*e.g.*, increased salivary cortisol levels). In humans, a less diverse microbiome would dampen the HPA stress system. It is unsurprising since GF or antibiotic-depleted rodent models do not reflect the complex physiological variations of the gut microbiota in humans. Also, given the small sample size of this study ¹⁸⁶, the authors do not reveal strong correlations between gut microbiota composition in one-month-old infants and their cortisol levels. However, it still suggests that high abundance of *Bifidobacterium* and *Streptococcus* and low abundance of *Bacteroides* are linked to increased cortisol levels. At the genus level, higher abundances of *Staphylococcus* and *Prevotella* and lower abundance of *Lachnospiraceae* are associated with greater cortisol concentrations in the saliva of one-month-old infants ¹⁸⁶, allowing potential hypotheses as to the influence of specific commensal populations with the establishment of the HPA stress system in early postnatal life.

3.3 Antibiotic exposure in early life and implications for neurodevelopmental disorders

3.3.1 Antibiotic exposure and neurodevelopmental disorders in humans

Neurodevelopmental disorders (NDD) are a group of conditions which etiology is still unknown today, and are characterized by issues in brain developmental processes such as neuronal and synaptic formation, neuroplasticity, cognitive and motor deficits, and very frequently, social and anxiety impairments as well as stereotypies³⁵⁷. They include autism spectrum disorder (ASD), attention-deficits and hyperactivity disorder (ADHD), schizophrenia and intellectual disability disorder, among others³⁵⁸ and concerns 15% of the world population²²⁴. Today, ASD is one of the most common NDD, with a rising prevalence of 1.09/10000 to 436/10000 people worldwide³⁵⁸. Many patients with NDD, in particular ASD, display GI impairments, such as diarrhea/constipation, abdominal pain, associated with significant alterations in their gut microbiota^{224,359}. Of note, NDD children frequently show a lower diversity or absence of the commensal bacteria *Enterococcus faecalis*, *Lactobacillus rhamnosus* and *Bifidobacteria spp.*, as well as lower numbers of SCFA-producing species compared to neurotypical patients³⁵⁹ and increased levels of ‘harmful’ bacteria, such as *Escherichia* or *Salmonella* species of the *Proteobacteria* genus³⁶⁰. Knowing these frequent co-morbid conditions, increasing number of pre-clinical and clinical studies focused on the potential role of gut microbes on the onset and progression of NDDs^{361,362} (see **Table 1** below).

Table 1. Studies on early life antibiotic exposure and effects on physiology and neurobehavior in humans

No. of Study subjects	Age of exposure	Antibiotic type	Age of testing	Main findings	Ref.
9 non-treated vs. 9 treated children	2-9 days-old	Intravenous Ampicillin/ Gentamycin	4-8 weeks-old	↓ <i>Bifidobacteria</i> species numbers and diversity in antibiotic-treated infants at 4- and 8-wk old (e.g., <i>Bifidobacterium spp.</i>) <i>B. longum</i> “resists” to antibiotic exposure	363
4352 non-treated vs. 10 220 treated children	≤ 2 years-old	Oral Penicillins Cephalosporins Macrolides Sulfonamides	≤ 15 years old	↑ Risk of Celiac disease with ↑ numbers of antibiotic prescriptions and penicillins exposure ↑ Risk of ADHD with ↑ numbers of antibiotic prescriptions and penicillins exposure ↑ Risk of ADHD development with age, more markedly in ♂ ↑ Risk of Autism with penicillins in both ♂ and ♀ ↑ Risk of Learning disability after cephalosporin exposure	364
671 606 children: 72% antibiotic-exposed	≤ 2 years-old	Oral penicillins OR Other broad-spectrum antibiotics	≤ 15 years old	↑ Risk of Autism with penicillins or other broad-spectrum antibiotics	365
1498 non-treated vs. 1554 treated pre-term children	Maternal exposure 0-10 days-old	Erythromycin OR Erythromycin + Amoxicillin/Clavulanate	7 years old	↑ Cerebral palsy prevalence with either antibiotic, aggravated with combination of erythromycin + amoxicillin/clavulanate	366
483 459 exposed children	3 months pre-conception to delivery OR ≤ 2 years-old	Oral penicillin OR Other broad-spectrum antibiotics (non-specified)	≤ 11 years old	↑ Risk of Autism or ADHD after maternal antibiotic exposure or early life exposure Aggravated with no. of prescriptions and/or exposure duration during the given period	367
990 098 exposed children	Whole pregnancy OR ≤ 2 years-old	Oral broad- and narrow-spectrum antibiotics	0-18 years old	↑ Risks of sleep disorders, ADHD, conduct disorder, mood and anxiety disorders with antibiotic exposure in utero or in the first 2 years of life	368

526 children: 70% antibiotic-exposed	0-3.5 years-old	Any antibiotic type and administration mode	3, 7 and 11 years-old	↑ Risks of lower IQ, emotional issues, ADHD, depression and tendency to ↑ risk of social problems.	369
446 (84% antibiotic-exposed)	0-2 years-old	Any antibiotic type and administration mode	11 years-old	↑ Risks of lower IQ, ADHD-inattentive type diagnosis, overall cognitive impairments (<i>e.g.</i> , learning, executive problems), socio-emotional issues Earliest exposure in the first 2 years of life are correlated with poorer neurocognitive outcomes	370
25 781 twin siblings	0-2 years-old	Any antibiotic type and administration mode	7-12 years-old	↑ ADHD and ASD, but confounded with shared environment and genetics	371
14 non-exposed vs. exposed 52 pre-term children	Perinatal (maternal + child) exposure	Any antibiotic type and administration mode	4-5 years-old	↑ Risks of altered EEG power and attention deficits	372

As mentioned before, one of the factors that modifies the microbiota assembly is antibiotic exposure to broad-spectrum antibiotics, which occurs frequently in the perinatal period in humans, notably in the contexts of c-section delivery, pre-term labor or risks for neonatal and/or maternal infections around birth ¹⁹⁵. In the context of pre-labor with intact maternal membranes, children from mothers that received broad-spectrum antibiotics as a prophylaxis showed increased risks of cerebral palsy, a CNS disorder characterized by motor and balance impairments ³⁶⁶, linking the establishment of brain developmental function and behavior with maternal antibiotic exposure around birth. Several other cohort studies ^{369,370} and meta-analyses ^{373,374} revealed that early life antibiotic exposure was associated with increased risks of NDD, including ASD and ADHD. These reports demonstrated positive associations between both postnatal ^{369,370} and perinatal antibiotic exposure ³⁶⁷ with neurocognitive impairments (*e.g.*, lower IQ), emotional and affective deficits (*e.g.*, high emotional state and hyperactivity ³⁶⁹) and the development of such disorders. In particular, in a Swedish population-based cohort study ³⁶⁷, perinatal antibiotic exposure, either during pregnancy or in early neonatal life, was found to be associated with increased risks of ASD and ADHD in children, and were performed with antibiotic quantity, type and timing of exposure. Of the most prescribed antibiotics in this developmental period are the β -lactams *e.g.*, penicillin, ampicillin or amoxicillin, and this specific class of antibiotics were associated with higher odds of ADHD or autism ³⁶⁷. Therefore, human associations studies indicate that broad-spectrum antibiotic exposure during the perinatal period might be detrimental for neurobehavior, and might be a potential risk factor for the onset/progression of NDD. Nonetheless, no causal relationship has been made between antibiotic-induced dysbiosis and the risk of NDD in humans, and mechanistic insights are still scarce. In an attempt to discover a causal link between early life antibiotics and NDD, several pre-clinical studies have been focusing on the microbiota-gut-brain-behavior axis and the link with NDD.

3.3.2 Antibiotic exposure and neurodevelopment disorders in rodents

Antibiotic exposure and NDD-related neurobehavioral impairments. Multiple animal studies started to dissect the neurodevelopmental processes influenced by early life antibiotic exposure, and how they could relate to the development of NDD. Notably, antibiotic-induced depletion of the gut microbiota, which provides more translational relevance compared to the GF model, investigated the neurobehavioral processes and potential mechanisms behind the impact of microbial on neurodevelopment and brain function in general (see **Table 2**).

Table 2. Studies on early life antibiotic exposure and effects on physiology and neurobehavior in rodents

Rodent model	Age of exposure (Treatment duration)	Antibiotic treatment (Dosage)	Age of testing	Effect(s) on gut microbiota and gastrointestinal tract	Physiological and neurobehavioral effects	Ref.
MOUSE						
BALB/c	Maternal exposure E12.5-P21 (4 wk)	Penicillin V (31mg/kg)	6 wk old	↓ <i>Bacteroidetes/Firmicutes</i> ratio and ↑ <i>Proteobacteria</i> between 3 and 6 wk old	Altered BBB permeability in hippocampus and frontal cortex ↑ Neuroinflammation in frontal cortex ↓ Sociability and social novelty ↑ Defensive behavior in ♂ and ↑ <i>Avpr1b</i> expression in ♂ and ♀ ↓ Anxiety-like behavior in ♂ (EPM)	216
C57B/6J	Maternal exposure E9-E16	Antibiotic cocktail Neomycin (5 mg/ml); Bacitracin (5 mg/ml); Pimaricin (1.25 µg/ml)	8-10 wk old	↓ <i>Lactobacillales</i> and ↑ <i>Clostridiales</i> subcluster XIVa in offspring's gut microbiota	Transient ↓ in offspring locomotor and exploratory activities Cross-fostering rescues behavioral impairments	375
C57B/6J	Maternal exposure P1-P8	Penicillin V (31 mg/kg) <i>OR</i> Antibiotic cocktail Ampicillin (1 mg/ml); Vancomycin (0.5 mg/ml) Metronidazole (1 mg/ml) Ciprofloxacin (0.2 mg/ml) Imipenem (0.25 mg/ml)	P9-P19 (Early life behavior) 7-11 wk old (Adult behavior)	Based on results by <i>Leclercq et al. (2017)</i> ²¹⁶	Penicillin V ↓ <i>Bdnf</i> expression in hippocampus ↑ Compulsive-like and anxiety-like behaviors (Marble burying, EPM) ↓ Social recognition Antibiotic cocktail ↓ Pup nest orientation ↑ Compulsive-like and anxiety-like behaviors (Marble burying, EPM, LDB) ↓ Novel object recognition	217
C57BL/6N	Maternal exposure E15-P3	Ampicillin (0.6 mg/ml)	P3	↓ Circulating bacterial-derived PGNs	Sex-dependent ↓ in <i>Pglyrp1</i> , <i>Pglyrp3</i> , <i>Nod1</i> and <i>Tlr2</i> expression ↓ in synaptic-related genes <i>Bdnf</i> and <i>Met</i> expression	220

CD-1	Maternal exposure E15-P0 (~6 d.)	Penicillin V (31 mg/kg)	P21 6-7 wk old	Possible transient ↓ in α-diversity in ♂	Altered immune cell numbers in ♂ ↓ Hippocampal <i>Avpr1a/Avpr1b</i> expression in ♂ ↓ Defensive behaviors in ♂ ↓ Anxiety-like behavior in ♀ ↓ Sociability in ♂	214
C57B/6J	Maternal exposure Pre-conception -E14.5 (~6 wk)	Antibiotic cocktail <i>Pre-conceptional period</i> Vancomycin (50 mg/ml); Neomycin (100 mg/ml); Metronidazole (100 mg/ml) <i>Gestational period</i> Ampicillin (1 mg/ml); Ampicillin (1 mg/ml); Vancomycin (0.5 mg/ml); Neomycin (1 mg/ml)	E14.5 6-10 wk old (Behavior)	↓ α-diversity in maternal gut microbiota ↑ <i>Verrucomicrobiae</i> and <i>γ-Proteobacteria</i> in maternal gut microbiota	↓ Brain and serum maternal metabolites: IP, TMAV, 3-IS, TMAO and HIP ↓ Somatosensory processing in adult offspring ↓ Fetal thalamocortical axonogenesis, rescued by IP, TMAO, 5-AV, 3-IS and HIP	228
C57B/6J	Maternal exposure E14.5-P1	Vancomycin (0.5 mg/ml) <i>OR</i> Antibiotic cocktail Ampicillin (1 mg/ml); Vancomycin (0.5 mg/ml) Neomycin (1 mg/ml)	P21-P180	↑ Cecal weight in antibiotic cocktail-treated adult mice ↓ α-diversity and ↓ <i>Bacteroidetes/Firmicutes</i> ratio antibiotic cocktail-treated mice ↓ <i>Lactobacillus johnsonii</i> after antibiotic cocktail in ♀ ↑ <i>Lactobacillus murinus</i> after antibiotic cocktail in both sexes ↓ <i>Bacteroides thetaiotaomicron</i> in adolescent vancomycin-treated mice	↓ Brain inflammatory markers after immune challenge in antibiotic cocktail-treated mice. Sex-dependent alterations of several predicted microbial metabolic pathways, more markedly in antibiotic cocktail-treated mice than vancomycin-treated mice	219
NIH Swiss	P2-P9 (Neonatal) P12-P18 (PreWean) P21-P27 (PostWean)	Antibiotic cocktail Ampicillin (100 mg/kg); Vancomycin (50 mg/kg); Gentamicin (100 mg/kg); Imipenem (25 mg/ml)	P5-P11 (Early life behavior) P40-P45 (Adolescent behavior)	↓ α- and β-diversity for all treatment groups ↑ <i>Clostridium</i> taxa in adolescence and ↓ SCFA-producing bacteria	↓ Monocytes and granulocytes numbers until adulthood ↑ Cytokines levels until adulthood in ♀ Altered microglia morphology in tested adolescent but not adult mice	218

			P75-P81 (Adult behavior)		<p>↑ Myelin-related and ↓ <i>Bdnf</i> gene expression in tested adolescent after neonatal exposure</p> <p>↓ Myelin-related and <i>Oxtr</i> gene expression in tested adolescent ♀ after PreWean exposure</p> <p>↑ Anxiety-like and Compulsive-like behaviors in tested adolescents</p>	
BALB/c	P14-P21 (7 d.)	Penicillin V (33 mg/kg)	P42-P51	<p>↓ α-diversity, ↓ <i>Bacteroidetes</i> and ↑ <i>Proteobacteria</i> at P21 but partially restored at P42</p>	<p>↓ <i>Avpr1a</i> expression in hippocampus and <i>Avpr1b</i> in hippocampus, hypothalamus and frontal cortex</p> <p>Altered immune cell numbers more markedly in ♂</p> <p>↓ BBB permeability in ♂</p> <p>↓ Sociability in ♂</p> <p>↓ Anxiety-like behavior in ♂ (EPM)</p>	215
NIH Swiss	P21 (4-7 wk)	Antibiotic cocktail: Ampicillin (1 mg/ml); Vancomycin (5 mg/ml); Neomycin (10 mg/ml); Amphotericin-B (0.1 mg/ml); Metronidazole (10 mg/ml)	7-11 wk old	<p>↑ <i>Bacteroidetes/Firmicutes</i> ratio</p> <p>↓ General bacterial diversity</p>	<p>↓ Novel object recognition</p> <p>↓ Anxiety-like behavior</p> <p>↓ Social cues processing (social transmission of food preference)</p> <p>Alterations of the Tryptophan/Kynurenine pathway</p> <p>↓ Expression of <i>Bdnf</i>, <i>Oxt</i> and <i>Avp</i> in hippocampus</p>	67
C57B/6J	P7-P23	Antibiotic cocktail Ampicillin (1 mg/ml); Vancomycin (1 mg/ml); Neomycin (0.5 mg/ml)	6 wk old	<p>↓ α- and β-diversity and ↑ <i>Bacteroidetes/Firmicutes</i> ratio in gut microbiota</p> <p>↓ Ileum barrier permeability and TLR gene expression, rescued by butyrate exposure</p> <p>↓ Fecal SCFA levels, partly rescued by butyrate exposure</p>	<p>↓ <i>Bdnf</i> expression and Neurogenesis in hippocampus</p> <p>↑ Myelin-related gene expression in PFC and hippocampus and Overproduction of myelin sheath</p> <p>↓ Exploratory behavior and Anxiety-like behavior</p> <p>Restoration of behavior and myelination by butyrate exposure</p>	232

C57Bl/6OlaH	Adolescence (P28-49) OR Adulthood (P76-97)	Antibiotic cocktail Ampicillin (1 mg/ml); Vancomycin (0.5 mg/ml); Metronidazole (1 mg/ml); Ciprofloxacin (0.2 mg/ml); Imipenem (0.25 mg/ml)	Adolescence (P73-96) OR Adulthood (P121-145)	↓ Bacterial richness and <i>Bacteroidetes/Firmicutes</i> ratio in cecal microbiota of adolescent mice sustained to adulthood	More marked molecular and neurobehavioral effects in mice treated in adolescence: ↑ Anxiety-like behaviors (EPM) ↑ Expression of genes involved in immune markers (<i>e.g.</i> , <i>Tlr4</i>), microglia activation, GABA and GLU neurotransmission, synaptic-related genes (<i>e.g.</i> , <i>Syp</i> , <i>Psd-95</i>), SCFA receptors in amygdala Altered BBB permeability	376
Thy1- α Synuclein Parkinson's model	5-6 wk old (7 wk)	Antibiotic cocktail: Ampicillin (1 mg/ml); Vancomycin (5 mg/ml); Neomycin (5 mg/ml); Gentamicin (100 mg/ml); Erythromycin (10 mg/ml)	12-13 wk old	↓ General bacterial diversity ↓ Gut motility	Rescue of neurodegeneration-induced locomotor deficits ↓ Microglia diameter in the CPu and SN.	377
C57BL/6	6 wk old (14 d.)	Ampicillin/Streptomycin /Clindamycin (1 mg/ml)	8 wk old	↓ <i>Bacteroidetes/Firmicutes</i> ratio ↑ <i>Enterobacteriaceae</i> taxon Altered gut endocannabinoid signaling	↓ BDNF in hippocampus ↓ Firing patterns of mPFC neurons ↓ Microglia and astrocytes in PFC and hippocampus ↑ Depressive-like behaviors	378
C57BL/6	8 wk old (7 wk)	Antibiotic cocktail: Ampicillin/Subalctam (1.5 mg/ml); Vancomycin (0.5 mg/ml); Ciprofloxacin (0.2 mg/ml); Imipenem/Cilastatin (0.25 mg/ml); Metronidazole (1 mg/ml)	13-15 wk old	↓ General bacterial diversity	↓ Novel object recognition ↓ Hippocampal neurogenesis Antibiotic-induced effects reversed by physical exercise or probiotics and/or Ly6C ^{hi} monocytes.	96
BALB/c	8-10 wk old (7 d.)	Antibiotic cocktail: Neomycin (5 mg/ml); Bacitracin (5 mg/ml); Pimaricin (1.25 μ g/ml)	8-10 wk old	↓ <i>Bacteroidetes/Firmicutes</i> ratio	↓ BDNF in amygdala ↑ BDNF in hippocampus ↓ Anxiety-like behavior (Dark-light box and elevated platform tests)	65

C57B/6J	8-11 wk old (11 d.)	Antibiotic cocktail Vancomycin (1.44 mg/ml); Neomycin (24 mg/ml); Bacitracin (24 mg/ml); Ampicillin (9.6 mg/ml) Meropenem (4.8 mg/ml)	11 wk old	↓ α -diversity in colonic gut microbiota ↓ colonic SCFA levels	↑ Serum corticosterone ↓ Serum p-cresol and TMAO Altered BBB permeability Altered <i>Bdnf</i> , <i>Nmda2b</i> , <i>Sert</i> and <i>Npy</i> brain region-specific expression ↓ Novel object recognition	379
RAT						
Wistar	Maternal exposure Preconception-E15 (6 wk)	1% of non-absorbable Succinyl-sulfa-thiazole in diet.	6-7 wk old	?	↓ Social interactions ↓ Anxiety-like behavior (EPM) Delayed sensorimotor gating	380
Sprague-Dawley	P4-P13 (10 d.)	Vancomycin (10, 30 or 100 mg/kg) <i>OR</i> Antibiotic cocktail: Neomycin (50-100 mg/kg); Bacitracin (50-100 mg/kg); Pimaricin (2.5-5 mg/ml)	4-11 wk old	↑ <i>Bacteroidetes/Firmicutes</i> ratio (4 wk). Recovery at 8 wk.	Dose-dependent ↑ in visceral hypersensitivity (4 and 11 wk) ↓ Splenic neutrophils numbers	205
Sprague-Dawley	9 wk old (13 wk)	Antibiotic cocktail: Ampicillin (1 mg/ml); Vancomycin (0.5 mg/ml); Ciprofloxacin (20 mg/ml); Imipenem (250 mg/ml); Metronidazole (1 mg/ml)	22 wk old	Altered gut microbiota richness and composition	Altered expression of miRNAs involved in axon guidance, oxytocin signaling and neurotrophic factors pathways in amygdala and PFC	381
Sprague-Dawley	10 wk old (6 wk)	Antibiotic cocktail: Ampicillin (1 mg/ml); Vancomycin (0.5 mg/ml); Ciprofloxacin (20 mg/ml); Imipenem (250 mg/ml); Metronidazole (1 mg/ml)	17-22 wk old	↓ <i>Bacteroidetes</i> and <i>Firmicutes</i> at 3 mo. and 4 wk old	↑ <i>Bdnf</i> expression in amygdala ↓ <i>Nr3c1</i> and <i>Crhr1</i> in hippocampus and amygdala Altered 5-HT concentrations ↓ Spatial memory ↑ Visceral hypersensitivity and Depressive-like behaviors	382

Antibiotic-induced microbiota depletion in early life leads to the impoverishment of beneficial bacteria, such as *Lactobacillaceae* and *Bacteroidaceae*²³², and favors the implantation of detrimental bacteria such as *Clostridiales* or *Enterobacteriaceae*, for example^{218,232,375}, which is clinically relevant given the high represented levels of *Clostridium* species in ASD patients³⁶⁰. Moreover, even a transient course of antibiotics during weaning or adolescence induces enduring gut microbiota alterations^{218,376}, thus questioning the potential effects on intestinal physiology and neurobehavior during adolescence. At the intestinal level, pre-weaning antibiotics affects gut permeability without inducing peripheral inflammation²³², but can increase the neuroinflammatory status and BBB permeability in the amygdala³⁷⁶. By contrast, postnatal antibiotic depletion leads to altered microglial morphology in the BLA of adolescent mice²¹⁸.

At the neurobehavioral level, these studies indicate that perturbations of the gut microbiota impact neuronal growth factors levels or expression, such as BDNF or synaptic-related proteins³⁷⁶, in socially- and emotionally-relevant brain regions like the hippocampus²¹⁷, amygdala^{65,382} and medial PFC^{232,379}, and leads to increased motor activity⁶⁵ and memory³⁷⁹. Likewise, depletion of gut microbial communities by antibiotics leads to HPA axis hyperactivity, at least partly explaining social and anxiety-like behavioral deficits^{379,382}. Depending on the defined postnatal time window during which antibiotics are administered in mice and the time of behavioral testing, anxiety-like, compulsive-like and social behavioral outcomes are different. Indeed, *Lynch and colleagues*²¹⁸ revealed that a pre-weaning antibiotic course, either directly or indirectly through the mother²¹⁷, results in a transient increase in anxiety-like behavior in adolescence, but not in adults, which are more marked in females compared to males. Compulsive-like behavior was evident following an early and pre-weaning postnatal treatment and sustained until adulthood, whereas no remarkable changes were observed in sociability and social novelty behaviors²¹⁸. Of note, depressive-like behavior and locomotor activity were not impacted by early life antibiotic treatment²¹⁸, although another study showed that prenatal antibiotic exposure leads to motor deficits in offspring at puberty but not later in adulthood³⁷⁵.

These neurobehavioral outcomes could be explained by several potential microbiota-gut-brain signaling pathways including neuroimmune and metabolic pathways. For instance, early life antibiotic depletion of the gut microbiota in mice leads to long-lasting changes in the innate immune cells numbers, including decreased numbers of circulating monocytes and granulocytes until weaning, and increased IFN- γ plasma levels up to adulthood²¹⁸. Of note, these cells participate to brain development³⁸³ and neuroimmune disturbances following

antibiotic exposure in early life might be at stake for the onset or progression of NDD. Likewise, antibiotic-induced microbiota depletion is detrimental for the serotonin signaling pathway along the microbiota-gut-brain axis, as L-tryptophan levels, the precursor amino acid of 5-HT, and 5-HT central metabolism are modified following antibiotic exposure³⁸². Likewise, GABA receptors and glutamate metabotropic receptor 5 are upregulated in the amygdala of mice treated with antibiotics in adolescence³⁷⁶, suggesting that gut microbiota alterations during this critical period of development might be a risk factor for abnormal neurotransmitter signaling later in life. Another potential signaling pathway at risk in NDD may be microbial metabolites including SCFAs, *p*-cresol and trimethylamine-N-oxide, which are all decreased in adult³⁷⁹ and post-weaned²³² depleted animals, but increased SCFA receptors gene expression can be found in the amygdala³⁷⁶, perhaps to compensate the decreased SCFA gut levels. In a pioneer study from *Vuong and colleagues*²²⁸, thalamocortical axonal formation and maternal microbial metabolites are impaired in fetal life in dams whose microbiota was depleted with an antibiotic cocktail, and results in significantly altered sensory processing in offspring. These somatosensory impairments are linked to decreased maternal microbial metabolites such as imidazole propionate, 3-indoxyl sulfate, trimethylamine-N-oxide and hippurate that promote specifically axonogenesis between the thalamus and the cortex. This important study²²⁸ thus suggests that these microbial-derived metabolites are transferred from the mother to the fetus from early to mid-gestation periods, and act as ‘neurotrophic’ factors for axonal developmental processes governing somatosensory development during this period. This also provides a potential mechanism by which maternal gut microbiota is paramount for fetal brain development and potential NDD onset later in life. Moreover, the myelin formation occurs mainly around the weaning period²²², and pre-weaning antibiotic treatment leads to the overproduction of the myelin sheath in the medial PFC, due to increased differentiation and maturation of oligodendrocytes in this brain region²³² and upregulation of myelination-related genes in the brain following pre-weaning exposure³⁷⁶, or at least a trend after postnatal antibiotic exposure²¹⁸. However, depending on the antibiotic course, duration and type in the pre-weaning period, there can be female-specific downregulation of myelination-related genes in the PFC²¹⁸. Importantly, hippocampal neurogenesis²³², cognitive deficits and decreased anxiety-like behavior are likely related to myelin formation defects.

However, the presented studies need to be interpreted carefully as most of them are conducted in adult animals^{65,379} and are administered through gavage^{232,379}, which can bias the

interpretation of stress-induced behavioral outcomes. Importantly, the reported antibiotic dosage are usually higher than what is clinically used.

Clinically-relevant antibiotic exposure in rodents and risk for NDD. Antibiotic-induced depletion is not translatable to humans, and the precise timing of exposure and antibiotic dosage need to be clearly considered in this purpose. Only few rodent studies ²¹⁴⁻²¹⁶ have investigated clinically-relevant doses of broad-spectrum antibiotics (administered at 31 to 33 mg/kg/day in the drinking water of mothers) in specific early developmental time windows on the development of the microbiota-gut-brain axis and their relevance for NDD (see **Table 2**). First, *Leclercq and colleagues* ²¹⁶ demonstrated that perinatal administration of low-dose penicillin induces offspring dysbiosis until adolescence, with a remarkable increase in *Proteobacteria* (e.g., *Escherichia* species) and an imbalanced *Bacteroidetes/Firmicutes* ratio. Gut microbiota alterations are associated to significant reductions in sociability and social interest in adolescent offspring, sex-dependent changes in anxiety-like behaviors and increased male aggression. In relation to behavioral outcomes, perinatally-exposed offspring display increased inflammation specifically in the frontal cortex, and an altered BBB integrity in both the hippocampus and frontal cortex ²¹⁶. This study thus indicates that even low-dose penicillin administered results in ASD-relevant behavioral abnormalities. Importantly, significant neuroinflammation and BBB integrity changes in social decision-making brain areas would contribute to these neurobehavioral impairments later in life. However, the antibiotic treatment duration in this study would be equivalent to a continuous course between the last trimester of pregnancy until childhood in human mothers ²²², which hardly corresponds to the clinical reality. A more clinically-relevant study investigated the impact of penicillin in the prenatal period (*i.e.*, last week of pregnancy) ²¹⁴, that lead to transient and sex-dependent changes in gut microbiota diversity, especially in exposed male offspring. At the behavioral level, anxiety-like behavior is reduced in exposed females whereas decreased social motivation and increased resilience to an aggressive competitor are observed in exposed males. These marked behavioral impairments in males are associated with a decreased number of Tregs and an increased number of inflammatory monocytes, suggesting a potential neuroimmune pathway mediating ASD-related behavioral impairments ²¹⁴. Compared to perinatal penicillin exposure ²¹⁶, prenatal penicillin induces sex-dependent, and most importantly, transient changes in the gut microbiota. Similarly, direct postnatal penicillin treatment between P14 and P21 disturbs the gut microbiota composition ²¹⁵, and is associated to reduced social behavior and increased BBB permeability in the hippocampus and frontal cortex in males, but not females. By contrast, indirect maternal

penicillin administration in the first week of life leads to increased compulsive-like behavior, decreased anxiety-like behavior and impaired social recognition in adult male offspring²¹⁷. Immune activation seems also to be triggered by direct postnatal antibiotic exposure, in males only²¹⁵. Similar to prenatal penicillin treatment²¹⁴, penicillin administered directly to pre-weaned offspring leads to relatively transient alterations in the gut microbiota²¹⁵. However, the effect of low-dose penicillin in this narrow postnatal period, corresponding to the peak of synaptic pruning and myelination processes in the mouse brain, has long-term effects on immune and neurobehavioral outcomes in mice²¹⁵. Altogether, these studies provide important insights into the impact of maternal and/or early life antibiotic exposure on brain development and the risk of NDD: (1) clinically-relevant doses of broad spectrum antibiotics in early life might be a risk factor for NDD-related behavioral impairments *i.e.*, social deficits, anxiety-like behavior and cognitive impairments, (2) there are variations in neurobehavioral outcomes depending on the timing of antibiotic exposure and dosage in early life and (3) sex-dependent changes in physiology and behavior reveal a male brain vulnerability, which is relevant to the sexually biased prevalence in ASD and ADHD.

Antibiotic exposure, neuropeptidergic systems and the risk for NDD. Specifically, exposure to broad-spectrum antibiotics may influence the developmental trajectories of the OXT/OXTR and AVP/AVPR1a/AVPR1b systems (see **Table 2**). For instance, a low dose of penicillin leads to decreased *Avpr1b* gene expression in the frontal cortex after prenatal exposure²¹⁴, and in the frontal cortex, hippocampus and hypothalamus, following postnatal exposure²¹⁵. By contrast, perinatal penicillin exposure between E12.5 and P21 increases *Avpr1b* expression in the frontal cortex, resulting in increased male defensive behavior towards an aggressor, and reduced sociability in antibiotic-exposed males, but not in females²¹⁶. The adolescent period is also a window of vulnerability for the OXT/OXTR and AVP/AVPR1a/AVPR1b systems towards the gut microbiota^{67,348}. Moreover, both OXT and AVP contribute to the regulation of the stress response along the HPA axis, and acute stress indeed combines with antibiotic effects to decrease hypothalamic *Oxt* mRNA levels, whereas *Avp* gene expression is reduced following antibiotic exposure independently from stress, leading to stress-induced socio-emotional deficits, such as decreased social activity, increased anxiety-like behavior and cognitive deficits⁶⁷, which resembles the core behavioral symptoms of ASD. In adulthood, antibiotic depletion and stress promote social avoidance and the activation of CRH neurons in the PVN and decreased GR-expressing cells in HPA axis regulating brain areas, such as the PVN, the BNST and the DG. These stress-induced social and anxiety-like symptoms are also observed in

antibiotic-depleted adults, when showing higher *Crh* mRNA and protein expression in the hypothalamus at basal levels, inducing increased PVN cell activity and hyperactivation of the HPA axis overall ⁹⁰. Interestingly, these results are in accordance to GF data in the same conditions ^{62,355}, again indicating that microbial colonization in early life is pivotal for the HPA axis normal activity and proper development of related socio-emotional behaviors. By contrast, antibiotic depletion in adult rats attenuates the stress-induced HPA axis hyperactivity, characterized notably by increased numbers of PVN GR-positive neurons and decreased *Crhr1* gene expression in stressed rats ³⁸⁴, indicating a potential species- and age-specific regulation of the stress response by intestinal microbes. Consequently, disturbing microbial colonization would have a negative impact over OXT and AVP developmental trajectories, and thus the establishment of socio-emotional and stress behaviors in rodents. Nonetheless, effects of antibiotic-induced perturbations of the gut microbiota on these systems depend on multiple factors such as the timing of exposure, the dose, the nature of the antibiotic molecule and the duration of treatment, thus forcing caution about results interpretation.

Thesis objectives

As mentioned in the general introduction, several birth-related conditions, such as c-section or pre-term delivery, require antibiotic prophylaxis in the mother and/or the child. Epidemiological studies have indicated a link between early life antibiotic exposure and adverse health outcomes later in life, including gut microbiota alterations and NDD-related neurobehavioral impairments. These studies have suggested a connection between antibiotics exposure around birth, alterations of microbial seeding and the establishment of NDD, but a causal relationship has not been demonstrated yet.

In parallel, rodent studies started to delineate the effect of antibiotics on maternal and offspring's gut microbiota, brain and behavioral components, focusing on various developmental periods during early life. However, high doses, the duration of antibiotic exposure and/or the combination between multiple antibiotic molecules in current pre-clinical antibiotic reports are not translatable to humans. For example, Leclercq and colleagues²¹⁶ investigated the physiological and neurobehavioral effects of a clinically-relevant dose of penicillin, the most commonly prescribed antibiotic drug in early life, but the duration of exposure (*i.e.*, mid-gestation until weaning) does not reflect human clinical conditions.

Moreover, brain developmental milestones between rodents and humans are temporally different. For example, postnatal day (P) 7 in mice is equivalent to birth in humans in terms of brain development, forcing caution about the choice of the studied developmental window in rodent models and its biological relevance to humans. In addition, the few studies investigating antibiotic-induced dysbiosis on offspring brain development and function only provided an overview of the effects on neonatal and juvenile behavior, especially social communication (*e.g.*, the neonatal ultrasonic vocalizations paradigm), representing one aspect of the pup's ability to bond and communicate with its mother. As the diagnosis of NDD often occurs in childhood, investigating socio-emotional behaviors and related brain molecular processes at the juvenile period would give an important insight into the possible effect of perinatal antibiotics on abnormal neurodevelopment.

In light of these observations, the objectives of this thesis is to study whether disturbances of the maternal gut microbiota with low-dose ampicillin would impact offspring's gut microbiota colonization and establishment later in life, impair NDD-relevant brain and behavioral outcomes in early life (*e.g.*, social, anxiety-like and cognitive behaviors), and if these behavioral

impairments are associated with inflammation and disruption of physiological barriers within the microbiota-gut-brain axis (see **Figure 17** below). Moreover, as the oxytocinergic and vasopressinergic systems are critically involved in the regulation of these behaviors, we hypothesized that potential antibiotic-induced behavioral impairments would interfere with the expression of neuropeptides and their receptors in brain regions controlling socio-emotional behaviors.

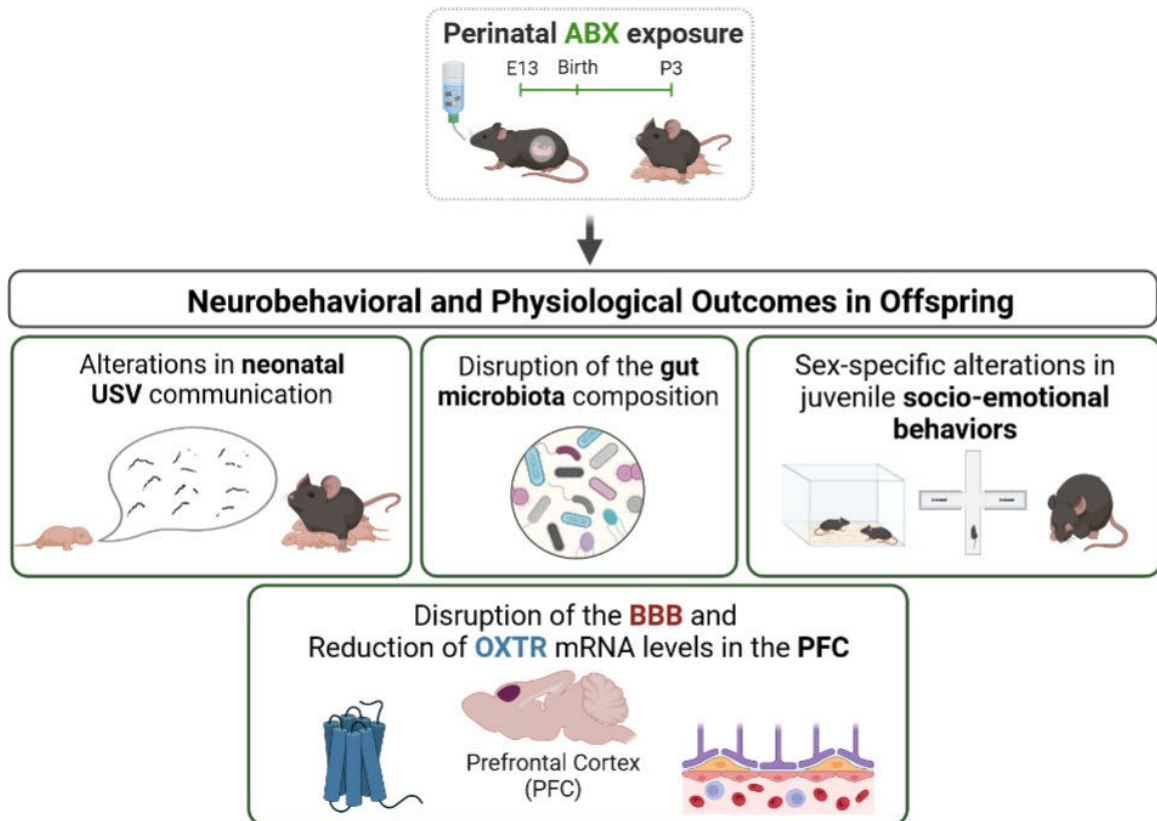
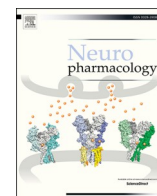


Figure 17. Graphical abstract of the results presented in C. Morel et al., 2023 (Neuropharmacology). In this study, maternal antibiotic (ABX) exposure with ampicillin in the perinatal period [i.e., embryonic day (E) 13 and P3] leads to various physiological and neurobehavioral outcomes in the neonatal and juvenile periods of the offspring. In particular, perinatal ampicillin treatment results in maternal and offspring's disruption of the gut microbiota composition. In the neonatal period, perinatally-exposed offspring mice showed altered social ultrasonic (USV) communication. At the juvenile period, perinatally-exposed offspring display sex-specific alterations in socio-emotional behaviors, notably in the free social interaction tests and elevated-plus maze. These behavioral impairments are associated with the disruption of the blood brain barrier (BBB) and a significant reduction in the oxytocin receptor (OXTR) in the prefrontal cortex (PFC).

Results - Publication



Perturbation of maternal gut microbiota in mice during a critical perinatal window influences early neurobehavioral outcomes in offspring

Cassandre Morel^{a,b}, Ines Martinez Sanchez^a, Yamina Cherifi^b, Nicolas Chartrel^b, Rochellys Diaz Heijtz^{a,*}

^a Department of Neuroscience, Karolinska Institutet, 171 77, Stockholm, Sweden

^b University of Rouen Normandy, INSERM, NorDIC, UMR, 1239, F-76000, Rouen, France

ARTICLE INFO

Handling Editor: Prof. J.F. Cryan

ABSTRACT

The gut microbiota is increasingly recognized as a key environmental factor that shapes host development and physiology, including neural circuits formation and function. Concurrently, there has been growing concern that early-life antibiotic exposure may alter brain developmental trajectories, increasing the risk for neurodevelopmental disorders such as autism spectrum disorder (ASD). Here, we assessed whether perturbation of the maternal gut microbiota in mice during a narrow critical perinatal window (last week of pregnancy and first three postnatal days), induced by exposure to a commonly used broad-spectrum oral antibiotic (ampicillin), influences offspring neurobehavioral outcomes relevant to ASD. Our results demonstrate that neonatal offspring from antibiotic-treated dams display an altered pattern of ultrasonic communication, which was more pronounced in males. Moreover, juvenile male, but not female, offspring from antibiotic-treated dams showed reduced social motivation and social interaction, as well as context-dependent anxiety-like behavior. However, no changes were observed in locomotor or exploratory activity. This behavioral phenotype of exposed juvenile males was associated with reduced gene expression of the oxytocin receptor (OXTR) and several tight-junction proteins in the prefrontal cortex, a key region involved in the regulation of social and emotional behaviors, as well as a mild inflammatory response in the colon. Further, juvenile offspring from exposed dams also showed distinct alterations in several gut bacterial species, including, *Lactobacillus murinus*, and *Parabacteroides goldsteinii*. Overall, this study highlights the importance of the maternal microbiome in early-life, and how its perturbation by a widely used antibiotic could contribute to atypical social and emotional development of offspring in a sex-dependent manner.

This article is part of the Special Issue on "Microbiome & the Brain: Mechanisms & Maladies".

1. Introduction

It is now widely recognized that the microorganisms inhabiting our gastrointestinal tract—the gut microbiota—modulates many aspects of host development and physiology, including the early life programming of brain circuits involved in motor control, emotion regulation, and cognition (for a review, see (Cryan et al., 2019)). Previous studies using germ-free (GF) mice have demonstrated that the gut microbiota influences a wide-range of neurodevelopmental processes, including blood-brain barrier (BBB) formation and integrity (Braniste et al., 2014), microglial maturation and function (Erny et al., 2015; Thion et al., 2018), and myelination (Hoban et al., 2016), as well as complex behaviors including social and anxiety-like behaviors (Clarke et al., 2013;

Diaz Heijtz et al., 2011; Neufeld et al., 2011). Recent interdisciplinary studies have revealed that common perinatal insults (e.g., stress, preterm birth, drug exposure, and altered diet) can perturb the assembly and maturation of the gut microbiota that occurs in parallel with critical windows of postnatal brain development (Codagnone et al., 2019; Borre et al., 2014). Moreover, there is growing evidence pointing to complex interactions between host genetics, gut microbiota, and perinatal risk factors to program susceptibility to neurodevelopmental and psychiatric disorders later in life (Buffington et al., 2021; Cong et al., 2016; Firestein et al., 2019; Kelsey et al., 2021).

Beta-lactam antibiotics, such as ampicillin, are the most frequently prescribed drugs during pregnancy and early postnatal life in the Western world (Ledger and Blaser, 2013). Although this class of drugs

* Corresponding author.

E-mail address: rochellys.heijtz@ki.se (R. Diaz Heijtz).

<https://doi.org/10.1016/j.neuropharm.2023.109479>

Received 16 December 2022; Received in revised form 20 February 2023; Accepted 24 February 2023

Available online 2 March 2023

0028-3908/© 2023 Published by Elsevier Ltd.

has been considered safe, they reduce the diversity and bacterial load of the gut microbiota by inhibiting the synthesis of the bacterial cell wall—thus, raising major concerns about their use in early-life. A growing body of epidemiological evidence suggests that early-life antibiotic exposure could have detrimental effects on the long-term general health of infants (e.g., development of immune and metabolic diseases (Cox and Blaser, 2015; Marra et al., 2009);) and may also increase the risk of childhood behavioral and emotional problems (Lavebratt et al., 2019; Slykerman et al., 2019). These clinical observations are supported by experimental studies in rodents showing that early-life antibiotic exposure (albeit over a prolonged developmental period) can lead to behavioral alterations later in life (Keogh et al., 2021; Volkova et al., 2021; Leclercq et al., 2017). For instance, Leclercq and colleagues reported that prolonged exposure to a low-dose penicillin (from late pregnancy until the end of the weaning period) induces long-term effects in offspring gut microbiota, brain cytokines and social behavior (Leclercq et al., 2017). However, more recent studies have begun to delineate specific critical developmental windows in which disruption of optimal host-microbiota interactions can lead to atypical brain development and behavior (Champagne-Jorgensen et al., 2020; O'Connor et al., 2021; Lynch et al., 2023).

One key developmental window of vulnerability is the perinatal period since disturbances of the maternal gut microbiota, in response to perinatal challenges such as stress, infections, and altered diet during pregnancy, have been associated with abnormalities in neurodevelopment and behavior of offspring (Gur et al., 2019; Rincel et al., 2019; Kim et al., 2017; Hsiao et al., 2013; Buffington et al., 2016). We have previously shown that a relatively low dose of broad-spectrum antibiotic (ampicillin), during the last week of pregnancy and the first three days after birth, reduces gene expression of the autism risk gene *c-Met* and brain-derived neurotrophic factor (Arentsen et al., 2017), both implicated in the formation and modulations of brain circuits (Eagleson et al., 2017; Cohen-Cory et al., 2010), in the neonatal mouse striatum. In terms of brain development, the time window between the last week of pregnancy and the first three days after birth [i.e., embryonic day (E)13 to postnatal day (P)3] in rodents is considered roughly equivalent to the second and third trimester in humans (Chini and Hanganu-Opatz, 2021; Semple et al., 2013). However, the first major exposure to a complex microbiota occurs during birth, via vertical mother-to-infant microbial transmission, in both rodents and humans (for a review, see (Cowan et al., 2020)). Arentsen and colleagues have previously documented that antibiotic exposure (ampicillin) during pregnancy leads to perturbations in the composition of the maternal gut microbiota (Arentsen, 2017). To our knowledge, changes in ASD-relevant behaviors during the neonatal-prepubertal period due to antibiotic-induced perturbation of the maternal gut microbiota have been largely neglected in previous research. In the current study, we sought to investigate whether antibiotic-induced perturbation of the maternal gut microbiota, during the same critical perinatal window of vulnerability, influences offspring neurodevelopment and ASD-relevant behaviors. Since clinical studies have demonstrated that various signs of ASD begin to emerge in the first year of life (Maestro et al., 2005), we chose to examine various ASD-relevant behaviors in the offspring during the neonatal-prepubertal stage of mice including ultrasonic communication, motor, and social-emotional development. In preclinical animal models of ASD, neonatal ultrasonic vocalizations (USVs) have been studied as an early communicative behavior of the mother-pup dyad that is relevant to the core symptoms of ASD (Caruso et al., 2020). The prepubertal (juvenile) stage is typically considered between P18 to P27 in mice, analogous to early childhood in humans (Bell, 2018), Motivations for sociability (i.e., the tendency to seek social interaction) is more prominent during the prepubertal stage, whereas aggression and sexual behaviors become more prominent after the onset of puberty (Fairless et al., 2012; Panksepp and Lahvis, 2007). Sociability during the prepubertal period is particularly relevant to ASD given the difficulties children with ASD have with social interaction (Lord et al., 2020). The three-chambered

social approach task has been widely used to assess sociability in mouse models of ASD, as early as P19 (Fairless et al., 2012). Given the important role of oxytocin (OT) and arginine vasopressin (AVP) systems in social and affective behaviors and their implications in the pathophysiology of ASD (Meyer-Lindenberg et al., 2011; Hornberg et al., 2020; Caldwell and Albers, 2016; Guastella et al., 2010), we examined whether antibiotic-induced perturbation of the maternal gut microbiota would influence aspects of these systems. Additionally, we examined the expression of genes associated with intestinal gut barrier and BBB integrity (i.e., cytokines and tight-junction proteins) in the brain and colon.

2. Material and methods

2.1. Subjects

Pregnant C57BL/6Rj dams with a defined day of fertilization were purchased from Janvier-Labs (Le Genest, Pays de la Loire, France). On their arrival (embryonic day 12; E12), dams were housed individually in standard plastic cages (Makrolon Type III, Tecniplast, Buguggiate, Italy), under controlled temperature, humidity, and light (12:12 light-dark cycle) conditions at the Comparative Medicine Biomedicum (KM-B), Karolinska Institutet. Food and water were provided *ad libitum*. All experiments were conducted according to a protocol approved by the Ethics Committee on Animal Research, Stockholm North and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Antibiotic treatment

Pregnant dams were assigned to either antibiotic treatment or control sterile water. To perturbate the maternal gut microbiota, we used a single wide-spectrum oral antibiotic (ABX; ampicillin, cat. no. A8351, Sigma-Aldrich, St Louis MO, USA) that has minimal penetrance beyond the GI tract when administered orally and with food (Lafforgue et al., 2008). Moreover, ampicillin absorption is significantly decreased with food. Ampicillin (1 mg/ml; freshly made each day) was provided *ad libitum* in drinking water during the last week of pregnancy and first three postnatal days.

2.3. Experimental design

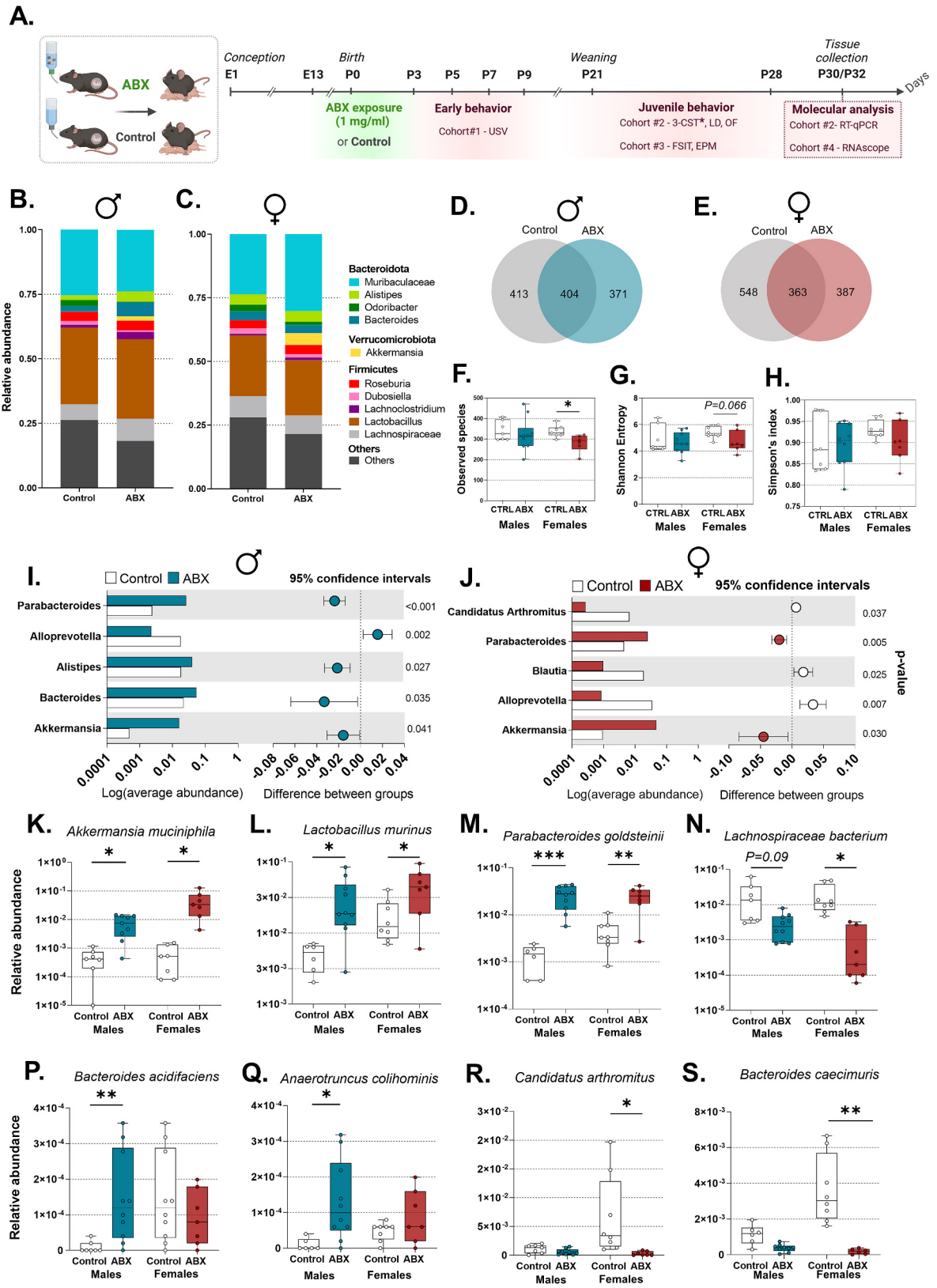
The full design of the study is illustrated in Fig. 1A. Briefly, four cohorts of animals from multiple independent litters (Cohort 1: n = 3–4 per group; Cohort 2: n = 7 per group; Cohort 3: n = 3–4 per group; Cohort 4: n = 3 per group) were used to assess the impact of perinatal ABX exposure on offspring neurodevelopment and behavior. The day of birth was considered as postnatal day (P)0. Offspring were weaned at P21 and randomly housed in same-sex and same-treatment groups (n = 3–5 mice per cage). Pup weight were measured across different developmental time points.

2.4. 16S rRNA metagenomic sequencing

DNA was extracted from mouse fecal samples using the QIAamp PowerFecal Pro DNA Kit (Qiagen AB, Sweden) according to the manufacturer's instructions. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified, sequenced by using Illumina Miseq 250 platform, and analyzed by Novogene (UK) Company, as previously described (Zhang et al., 2017).

2.5. Behavioral testing

Testing took place between 0900 and 1700 h under low illumination (40 Lux), except for the high-light open field (HOF) test. On the day of testing, animals were brought in their home cages to the testing room



(caption on next page)

Fig. 1. Experimental design and fecal microbiota composition of juvenile offspring from control and ABX-exposed dams. (A) Schematic representation of the study design. One week before delivery (embryonic day 13; E13), pregnant dams received either water (Control group), or a single broad-spectrum antibiotic (ABX: ampicillin; 1 mg/ml/day) in their drinking water. The ABX treatment was continued up to postnatal day 3 (P3). Perinatally ABX-exposed neonatal offspring were evaluated in the isolation-induced ultrasonic vocalizations paradigm (USV; cohort #1). After weaning, perinatally ABX-exposed juvenile offspring were subjected to a battery of behavioral tests: the three-chamber social approach task (3-CST; cohort #2) and the light-dark box test (LDB; cohort #2), the open-field test (OF; cohort #3), the free social interaction test (FSIT; cohort #3) and the elevated-plus maze (EPM; cohort #3). Feces (*) from cohort #2 were collected for fecal microbiota analysis. Brain and colon were collected at P30/P32 for gene expression (cohort #2) and RNAscope *in situ* hybridization analysis (cohort #4). (B, C) Relative abundance of bacterial genera in both males (B) and female (C) offspring from control and ABX-exposed dams. (D, E) Venn diagrams depicting the unique (single circle) and shared (circle overlap) operational taxonomic units (OTUs) in males (D) and females (E) from control and perinatally ABX-exposed juvenile offspring. (F–H) Bacterial alpha diversity was calculated using the following indices: (F) Observed species (species richness), (G) Shannon entropy (species evenness) and (H) Simpson's index (I, J). Relative abundance and 95% confidence intervals of bacterial genera showing significant changes in perinatally ABX-exposed male (I) and female (J) juvenile offspring compared to their respective control groups. (K–S) Relative abundance of bacterial species from the fecal microbiota of male (n = 7–9 per group) and female (n = 7–8 per group) offspring from control and ABX-exposed dams, including: *Akkermansia muciniphila* (K), *Lactobacillus murinus* (L), *Parabacteroides goldsteinii* (M), *Lachnospiraceae bacterium* (N), *Bacteroides acidifaciens* (P), *Anaerotruncus colihominis* (Q), *Candidatus arthromitus* (R) and *Bacteroides caecimuris* (S). Data in (K–S) represent means \pm SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control offspring mice.

and allowed to habituate for at least 1 h. Test chambers were first cleaned with 70% ethanol, and then water after each animal. All animals were naïve to the behavioral tests.

2.6. Isolation-induced ultrasonic vocalizations test

During the neonatal period, mouse pups emit ultrasonic vocalizations (USVs; frequencies between 30 and 90 kHz) when they are isolated from the nest to gain their mother's attention (D'Amato et al., 2005). In this study, isolation-induced USVs of pups were measured at P3, P5, P7 and P9 to determine the impact of ABX on social communicative behavior in early life (Grimsley et al., 2011; Scattoni et al., 2009). Briefly, pups were individually removed from the nest and placed into a clean plastic container directly under an ultrasonic-sensitive microphone (a condenser ultrasound microphone, CM16/CMPA, Avisoft Bioacoustics, Germany) that was connected to a recording interface (Ultrasound Gate 116Hb, Avisoft Bioacoustics), within a sound-proof box (SR-LAB Startle Response System, San Diego Instruments, CA, USA). The temperature inside the box was continuously monitored to prevent hypothermia. Pup vocalizations were recorded for 5 min. Total number of calls, duration of calls, frequency of calls and call characterization were monitored through the Avisoft Recorder USGH (version 4.2.16; Avisoft Bioacoustics) software and analyzed using the VocalMat automated software, developed by Dietrich's laboratory on MATLAB. This software is based on computational vision to process spectrograms of USVs, generated by the Avisoft Recorder, and machine learning to characterize the different types of calls in an automated manner (Fonseca et al., 2021).

2.7. Low-light open field (OF) test

Mice were placed individually in the center of the open-field box (48 cm \times 48 cm; ActiMot detection system; TSE, Bad Homburg, Germany), and their spontaneous motor activity was recorded for 30 min. The following parameters were analyzed: total distance travelled (meters), number of rears (*i.e.*, when an animal stands up on its hind legs), time spent (seconds) and distance travelled in the center of the OF arena. These parameters were automatically recorded by the Acti-Mot detection system.

2.8. High-light OF test

Animals were exposed to the OF test described above under bright lights (400 lux) for a period of 10 min.

2.9. Three-chambered social approach task (3-CST)

Social preference (*i.e.*, preference for a stimulus mouse over a novel object) was evaluated in a three-chambered apparatus, as previously described (Arentsen et al., 2017). After each animal, the top frame of the

apparatus was thoroughly cleaned with 70% ethanol and water, while its support base was cleaned with soap, water and 70% ethanol to remove odors from urine and feces. Time spent in each chamber (in seconds), time in close interaction (*i.e.*, the time spent sniffing and touching physically the enclosure; in seconds) and total distance travelled (in centimeters) were recorded and analyzed using an automated video-tracking system (EthoVision XT version 11, Noldus, Wageningen, The Netherlands). Time spent in close interaction with either the empty enclosure or the stimulus mouse enclosure was used to calculate the sociability index (SI; in %), as follows: $SI = [Ts / (Ts + Te)] * 100$ with Ts, the time spent in close interaction with the stimulus mouse, and Te, the time spent in close interaction with the empty enclosure (Antunes and Biala, 2012).

2.10. Light-dark box test

The light-dark box (LDB) test consisted of two equal-sized (24 cm \times 24 cm) Plexiglas compartments, in which one compartment was transparent and illuminated with a LED light strip (1600 lux) and the other compartment was black. A rectangular door in the middle wall connected the two compartments. In this test, mice were placed individually into the dark compartment to start the test and were then allowed to freely explore the apparatus for 5 min. The following parameters were analyzed in each compartment: time spent, distance travelled, and number of rears. These parameters were automatically recorded by the Acti-Mot detection system (TSE, Bad Homburg, Germany) using photo-beam cells.

2.11. Free social interaction test

The free social interaction test (FSIT) assesses active social interaction toward a novel, conspecific mouse. Two mice of the same age, weight, and sex were allowed to freely interact in a novel arena (30 cm \times 30 cm). On the day of experiment, the test mouse was habituated individually to the arena for 15 min before testing. The behavioral task started with the introduction of an unfamiliar stimulus mouse into the arena of the test animal. Social interactions were recorded for 5 min with a video camera (Samsung, Seoul, South Korea) placed in front of the cage (horizontal plane). The time the test mouse spent interacting with the stimulus mouse was manually scored. Active interaction was defined as sniffing (*i.e.*, anogenital, nose-to-nose, nose-to-head, and nose-to-body physical interactions), close following, and allo-grooming behavior.

2.12. Elevated plus maze

The test mouse was placed in the center of an elevated plus maze (EPM) apparatus, always facing the same open arm, and was allowed to explore all four arms for 5 min. The EPM apparatus (Kinder Scientific) was made of black Plexiglas and consisted of two open arms (36 \times 5 cm),

two enclosed arms (36 × 5 cm), and a central area (5 × 5 cm) that were elevated 64 cm above the floor level. Walls of the closed arms were 16 cm high and made of the same material. The following parameters were measured using infrared photo-beams: number of entries, time spent, and distance travelled in the open arms, closed arms, and intersection (center of the maze).

2.13. RNA extraction and quantitative real-time PCR

Total RNA was isolated from the hypothalamus, prefrontal cortex, amygdala, and colon of prenatally ABX-exposed juvenile offspring (i.e., at P28–P31 post-behavior), using the RNeasy® Mini Kit according to the manufacturer's instructions (Cat No. 74104, Qiagen, Sweden). RNA concentration and purity were quantified using the ND-1000 spectrophotometer (Nanodrop, ThermoFisher, Sweden). Following RNA extraction, 1 µg of each mRNA sample was reverse transcribed into cDNA using the iScript® cDNA synthesis kit (Cat.No. 1708890, Bio-Rad, Sweden). All cDNA samples were stored at –20 °C until further use. Gene expression was analyzed using the SYBR Green technology and primers specific to the genes of interest. Then, the qPCR assay was performed on the CFX384 Touch Real-Time PCR Detection System (Model #1855484, Bio-Rad, Sweden) and analyzed with the CFX Manager software (Version 3.1, Bio-Rad, Sweden). Reference genes were assessed *a priori* according to the type of tissue, sex, and treatment. Fold changes were calculated using the $\Delta\Delta C_t$ method. Primer sequences can be found in Table S1.

2.14. RNAscope *in situ* hybridization assay

Mice were deeply anesthetized and perfused transcardially with sterile 1X PBS followed by sterile 4% paraformaldehyde (pH 7.4) in 1X PBS. Brains were immediately removed, post-fixed overnight at 4 °C in sterile 4% paraformaldehyde and transferred consecutively to 15% and 30% sucrose solutions in 1X PBS (pH 7.4) at 4 °C. Next, 12-µm coronal fixed-frozen sections were obtained by cryostat sectioning and collected every 220 µm across the medial prefrontal cortex ([mPFC], distance from Bregma +1.04 mm to +2.44 mm; 6 sections/brain), according to the Allen Mouse Brain Reference Atlas (Allen Institute for Brain Science; www.atlas.brain-map.org/). Sections were mounted on SuperFrost Plus Slides (Thermo Fisher Scientific, Illkirch, France) and subsequently stored at –80 °C. Fluorescent *in situ* hybridization for the simultaneous detection of Oxt and Crhr1 transcripts was performed using the RNAscope Fluorescent Multiplex Reagent Kit V2 (Cat no. 323100; Advanced Cell Diagnostics [ACD], Rennes, France). The Oxt probe targeted the region 776–1676 (accession number NM_001081147; Cat no. 412171). The Crhr1 probe targeted the region 813–2072 (accession number NM_007762.4; Cat no. 418011-C3). Negative and positive control probes recognizing dihydrodipicolinate reductase, DapB (a bacterial transcript), Polr2A (C1 channel) and Ubc (C3 channel), respectively, were used to ensure tissue RNA integrity and optimal assay performance. Briefly, sections were submerged in 1X Target Retrieval (RNAscope Target Retrieval Reagents, Cat. No. 322000), heated at 98.5°C–99.5 °C for 5 min, followed by two washes of 2 min each. Then, they were quickly dehydrated in 100% ethanol and air dried for 5 min. The following day, Protease III was applied on top of each section for 30 min (RNAscope Pretreatment Reagents, Cat. No. 322381), followed by two washes of 2 min each. Probes mix was prepared as follows: Crhr1-C3 (Channel 3) was diluted 1:50 in Oxt-C1 (Channel 1), then hybridized for 2 h at 40 °C and washed twice in 1X Wash Buffer (Cat No. 310091). Amplification steps were performed by incubating with Amp1 (30 min), Amp2 (30 min), and Amp3 (15 min) at 40 °C. Slides were washed twice in wash buffer in-between steps. Then, for Oxt detection, sections were incubated with HRP-C1 for 15 min at 40 °C and washed twice in wash buffer for 2 min. Opal 650 fluorophore (Akoya Biosciences, Cat No. FP1496001KT) was diluted 1:1000 in RNAscope Multiplex TSA Buffer (Cat No. 322809) and incubated for 30 min at 40 °C followed by two

washes of 2 min, and by HRP blocker incubation for 15 min at 40 °C. The same procedure was performed for Crhr1 detection with HRP-C3, and the Opal 570 fluorophore (Akoya Biosciences, Cat No. FP1488001KT) diluted 1:1500 in TSA Buffer. Finally, slides were counterstained with RNAscope DAPI, coverslipped with Fluoromount Aqueous Mounting Medium (Sigma-Aldrich, Cat No. F4680) and stored in the dark at 4 °C until imaging.

2.15. Image acquisition and analysis

Image acquisition was performed on a Leica Thunder Imager 3D microscope (DM6 B; Leica, Paris, France). Each brain slice was first imaged at a 10X objective to confirm its rostro-caudal localization in the brain using anatomical landmarks from the Allen Mouse Brain Reference Atlas. Then, images were acquired at a 20X magnification to focus on the region of interest (i.e., the prefrontal cortex), and at 40X magnification to appreciate qualitatively Oxt and Crhr1 expression at the cellular level. Finally, images were processed in Fiji/ImageJ (version 1.53f51; National Institute of Health, Washington, DC, USA) and adjusted for identical settings of brightness and contrast.

2.16. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 9 software or R (version 4.1.2). Results are presented as means ± standard error of the mean (SEM). Significant outliers were identified using Grubb's test ($\alpha = 0.05$). Data from the USV, OF, 3CST, LDB and EPM were analyzed using either repeated-measures analysis of variance (ANOVA) or factorial ANOVA when appropriate. *Post hoc* comparisons were made using a Bonferroni/Dunn test when significant ANOVA effects were found. Data from the FSIT and gene expression results were analyzed using an unpaired *t*-test followed by Welch's correction. The Benjamini-Hochberg procedure was performed with a false discovery rate (FDR; *q*-value of 0.1 as cut-off) to correct for multiple testing in tests involving microbiome data. Litter effects were not investigated. The threshold for statistical significance was set as $p \leq 0.05$.

3. Results

3.1. Perinatal ABX exposure did not affect dam water intake or offspring body weight

Water intake of C57BL/6Rj dams from Cohorts 3 and 4 was monitored to estimate daily consumption of the ABX treatment. No differences in water intake were found between control and ABX-exposed mothers throughout the treatment period (Treatment effect: [$F_{(1, 169)} = 0.6762, p = 0.4120$]; Treatment x Gestational Day interaction: [$F_{(9, 169)} = 0.9344, p = 0.4968$]; Fig. S1A). However, there was a significant effect of Gestational Day [$F_{(9, 169)} = 26.13, p < 0.0001$] on water intake, as the weight of the pregnant dams was increasing from E15 to birth, and then pups were growing during the first three postnatal days.

Overall, the body weight of control and exposed offspring was similar during the neonatal period (P3–P9; Treatment effect: [$F_{(1, 131)} = 1.308, P = 0.2615$]; Sex effect: [$F_{(1, 131)} = 0.002145, P = 0.9634$]; Sex × Treatment interaction: [$F_{(1, 131)} = 0.5802, P = 0.4520$]); except at P9, when the male offspring from ABX-treated dams had a significantly lower body weight compared to their respective control group (Postnatal day x Treatment: [$F_{(3, 93)} = 4.588, P = 0.0048$]; Bonferroni's *post hoc* comparison: $p = 0.0274$; Fig. S1B). During the early postnatal phase (P14–P28), no significant differences in body weight were observed between perinatally ABX-exposed offspring and their respective control groups (Treatment effect: [$F_{(1, 104)} = 2.643, p = 0.1070$]; Age x Treatment: [$F_{(2, 104)} = 0.3860, p = 0.6808$]; Sex x Treatment: [$F_{(1, 104)} = 0.08208, p = 0.7751$]) Fig. S1C).

3.2. Perinatal ABX exposure induced alterations in gut microbiota composition of juvenile mice

The relative abundance of the 10 top taxa at the genus level of perinatally ABX-exposed male and female juvenile offspring and their respective control groups is shown in Fig. 1B and C. Venn diagrams depicting the unique and shared operational taxonomic units (OTUs) between perinatally ABX-exposed male and female juvenile offspring and their respective controls are shown in Fig. 1D and E. We found that the relative abundance of the genera *Parabacteroides*, *Alistipes*, *Bacteroides* and *Akkermansia* was significantly increased in the perinatally ABX-exposed male juvenile offspring compared to their respective control group (Fig. 1I). On the other hand, the genus *Alloprevotella* was significantly reduced in the perinatally ABX-exposed juvenile group (Fig. 1I). In the perinatally ABX-exposed female juvenile offspring, we observed a significant increase in the relative abundance of the genera *Parabacteroides* and *Akkermansia* (Fig. 1J), but a significant reduction in the relative abundance of *Candidatus Arthromitus*, *Blautia* and *Alloprevotella* (Fig. 1J).

The relative abundance of several gut bacterial species, including *Akkermansia muciniphila*, *Lactobacillus murinus*, and *Parabacteroides goldsteinii* were significantly increased in the perinatally ABX-exposed male and female juvenile offspring (Fig. 1K-M), while other species such as *Lachnospiraceae bacterium* were significantly reduced (Fig. 1N). Interestingly, the relative abundance of *Bacteroides acidifaciens* and *Anaerotruncus colihominis*, was significantly higher in the juvenile male offspring from ABX-treated dams (Fig. 1P-Q), while other species such as *Candidatus arthromitus* and *Bacteroides caecimuris* were significantly reduced in the perinatally ABX-exposed female juvenile offspring (Fig. 1R-S).

Alpha diversity (species diversity of a given community) was analyzed at the genus level. The number of observed species (richness) was significantly decreased in perinatally ABX-exposed female juvenile offspring (Fig. 1F). The Shannon entropy (evenness) was also decreased in this group, but it did not reach statistical significance ($p = 0.066$; Fig. 1G). A similar tendency was observed in the perinatally ABX-exposed male juvenile offspring (albeit without reaching statistical significance) (Fig. 1F and G).

3.3. Early-life social communication of male offspring is affected by perinatal ABX exposure

To assess the effect of a perinatal ABX exposure on early life social communication, we carried out an in-depth analysis of pup USVs. Overall, we found that the perinatal ABX exposure affected USV vocal parameters and repertoire, more markedly in the male offspring. Perinatally ABX-exposed males produced significantly more USV calls than matched controls at P7 (Treatment \times Age interaction: $[F_{(3, 63)} = 3.719, p = 0.0158]$; Bonferroni's *post hoc* comparison: P7 control vs. ABX males: $p = 0.0041$) (Fig. 2A) and the total duration of calls was also increased at P7 in perinatally ABX-exposed male offspring compared to matched controls (Age effect: $[F_{(3, 63)} = 6.520, p = 0.0007]$; Treatment \times Age interaction: $[F_{(3, 63)} = 2.298, p = 0.0861]$; Bonferroni's *post hoc* comparison: P7 control vs. ABX males: $p = 0.0306$) (Fig. 2C). In addition, the frequency of calls (in kHz) was significantly modulated by ABX exposure in male offspring (Treatment effect: $[F_{(1, 64)} = 15.91, p = 0.0002]$, and by the age of the tested pup (Age effect: $[F_{(3, 64)} = 22.67, p < 0.0001]$, with a reduction of the frequency at P7 and P9 (Treatment \times Age interaction: $[F_{(3, 64)} = 2.878, p = 0.0428]$; Bonferroni's *post hoc* comparison: P7 control vs. ABX males, $p = 0.0013$; P9 control vs. ABX males: $p = 0.0289$) (Fig. 2E).

In female offspring, there was a significant Age effect: $[F_{(3, 59)} = 11.62, p < 0.0001]$ on the total number of calls produced, which tended to be lower in perinatally ABX-exposed females at P9 (Treatment \times Age interaction: $[F_{(3, 59)} = 2.184, p = 0.0995]$; Fig. 2B). There was a significant Age effect on the duration of calls $[F_{(3, 59)} = 5.754, p =$

0.00016], but no significant effects of Treatment or Treatment \times Age (Fig. 2D). However, the frequency of calls was significantly affected by Age: $[F_{(3, 60)} = 6.671, p = 0.0006]$, Treatment: $[F_{(1, 60)} = 6.180, p = 0.0157]$ and Treatment \times Age interaction: $[F_{(3, 60)} = 3.478, p = 0.0213]$, with a significant reduction in call frequency in the perinatally ABX-exposed female offspring both at P7 and P9. Bonferroni's *post hoc* comparison: P7 control vs. ABX females: $p = 0.0133$; P9 control vs. ABX females: $p = 0.0485$) (Fig. 2F).

Next, we assessed the effect of the perinatal ABX treatment on the abundance of different call categories, together referred as the USV call repertoire (inset of Fig. 2). In both male and female offspring, the most abundant call categories were the downward and the step-up calls. In the male offspring, significant effects of perinatal ABX exposure on the abundance of different call categories occurred at P3 and P7. There was a significant decrease in step-up calls ($p = 0.0114$) in males from ABX-treated dams at P3 compared to their respective controls (Fig. 2G). In females, however, no changes were observed following ABX exposure at P3, except a tendency for a reduced number of downward calls (Bonferroni's *post hoc* comparison: control vs. ABX: $p = 0.0839$) (Fig. 2G). At P7, males displayed a significant increase in step-up and flat call number (Bonferroni's *post hoc* comparison: control vs. ABX males: $p < 0.0001$) (Fig. 2I), but no changes were observed in the female offspring at this age (Fig. 2I). Finally, the ABX treatment did not impact the USV call patterns at P5 (Fig. 2H) or P9 (Fig. 2J) in neither males nor females.

3.4. ABX-exposed male offspring showed social behavior impairments in the juvenile period

Social behavior was analyzed between P23–P26, using the three-chamber social approach task (3-CST; Fig. 3A–H) and the free social interaction test (FSIT; Fig. 3I–N). Representative tracks of movement patterns of controls and perinatally ABX-exposed male and female juvenile offspring during the sociability phase of the 3-CST are shown in Fig. 3G, H. Consistently with the results from the neonatal period (*i.e.*, P3–P9), the effect of the perinatal ABX exposure on social behavior was more pronounced in the male juvenile offspring. In the 3-CST, mice were given the choice to explore chambers containing either an age- and sex-matched stimulus mouse or an empty enclosure. Perinatally ABX-exposed male juvenile offspring had a higher mean latency to first approach the stimulus mouse compared to their respective control group, but this trend did not reach statistical significance (Treatment \times Chamber interaction: $[F_{(1, 34)} = 3.570, p = 0.0674]$) (Fig. 3A). However, there were no significant differences between the two groups with respect to the total time spent in the chamber containing the stimulus ($p > 0.1$; Fig. 3B) and time spent in close interaction with the stimulus ($p > 0.1$; Fig. 3C). On the other hand, we found that perinatally ABX-exposed female juvenile offspring spent significantly more time in close interaction with the stimulus mouse than their respective controls (control vs. ABX females: 31.89 ± 10.82 vs. 45.68 vs. 16.62 ; $p = 0.0413$; Fig. 3F), indicating higher sociability. Indeed, the sociability index of perinatally ABX-exposed female juvenile offspring was significantly higher than that of their respective controls (control vs. ABX females: 56.1 ± 5.5 vs. 73.7 ± 3.2 ; $p = 0.0213$). However, no significant differences were observed with respect to the latency to first approach ($p > 0.1$; Fig. 3D) or total time spent in the chamber containing the stimulus ($p > 0.1$; Fig. 3E).

In the FSIT, perinatally ABX-exposed male juvenile offspring spent significantly less time interacting with the stimulus mouse compared to their respective control group (Welch's *t*-test, $p = 0.0205$; Fig. 3I). They also had a lower number of physical contacts with the stimulus mouse ($p = 0.0395$) (Fig. 3K). Consistent with the 3-CST results, the latency to first contact with the stimulus mouse tended to be higher in the perinatally ABX-exposed male juvenile offspring ($p = 0.0858$) than their controls (Fig. 3J). By contrast, the perinatally ABX-exposed female juvenile offspring did not show any significant changes in the FSIT test (Fig. 3L–N).

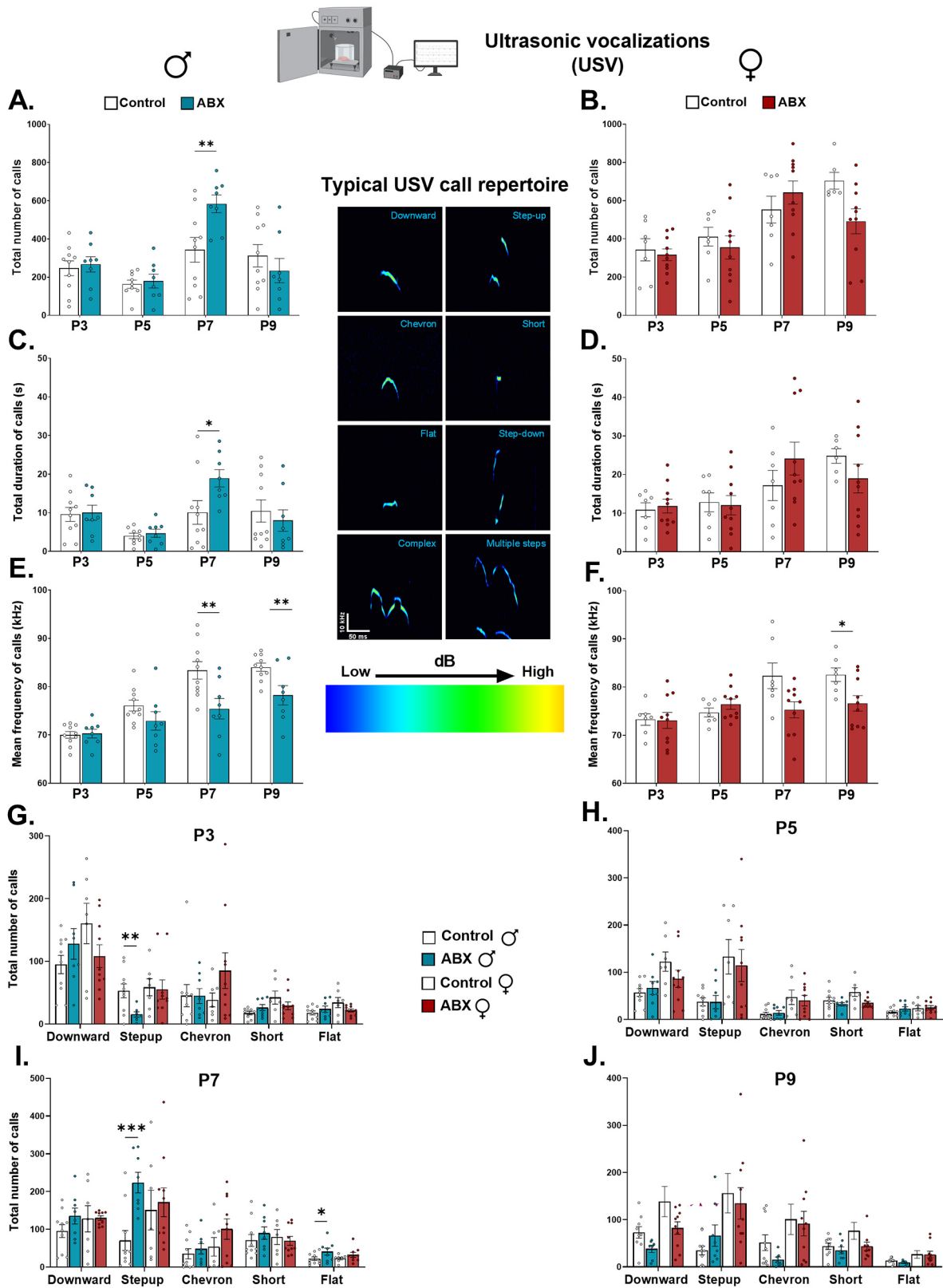


Fig. 2. Ultrasonic vocalizations (USV) features and repertoire of neonatal offspring are altered by perinatal ABX exposure. (A–F) Total number (A, B), total duration (in seconds) (C, D) and mean frequency of calls (in kHz) (E, F) of control and perinatally ABX-exposed male ($n = 8–10$ per group) and female ($n = 7–10$ per group) offspring, during the early postnatal period. (G–J) Proportions of calls per USV call category for each experimental timepoint at P3 and P5 (G, H) and P7 and P9 (I, J) of control and perinatally ABX-exposed male and female offspring. (Middle inset) Representative spectrograms of USV call repertoire emitted by neonatal offspring at each experimental timepoints. Time (in seconds) is represented on the x-axis and call frequency is indicated (in kHz) on the y-axis. Relative sound intensity (in dB) is represented by the color gradient scale. All data are presented as means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to control offspring mice. P, postnatal day.

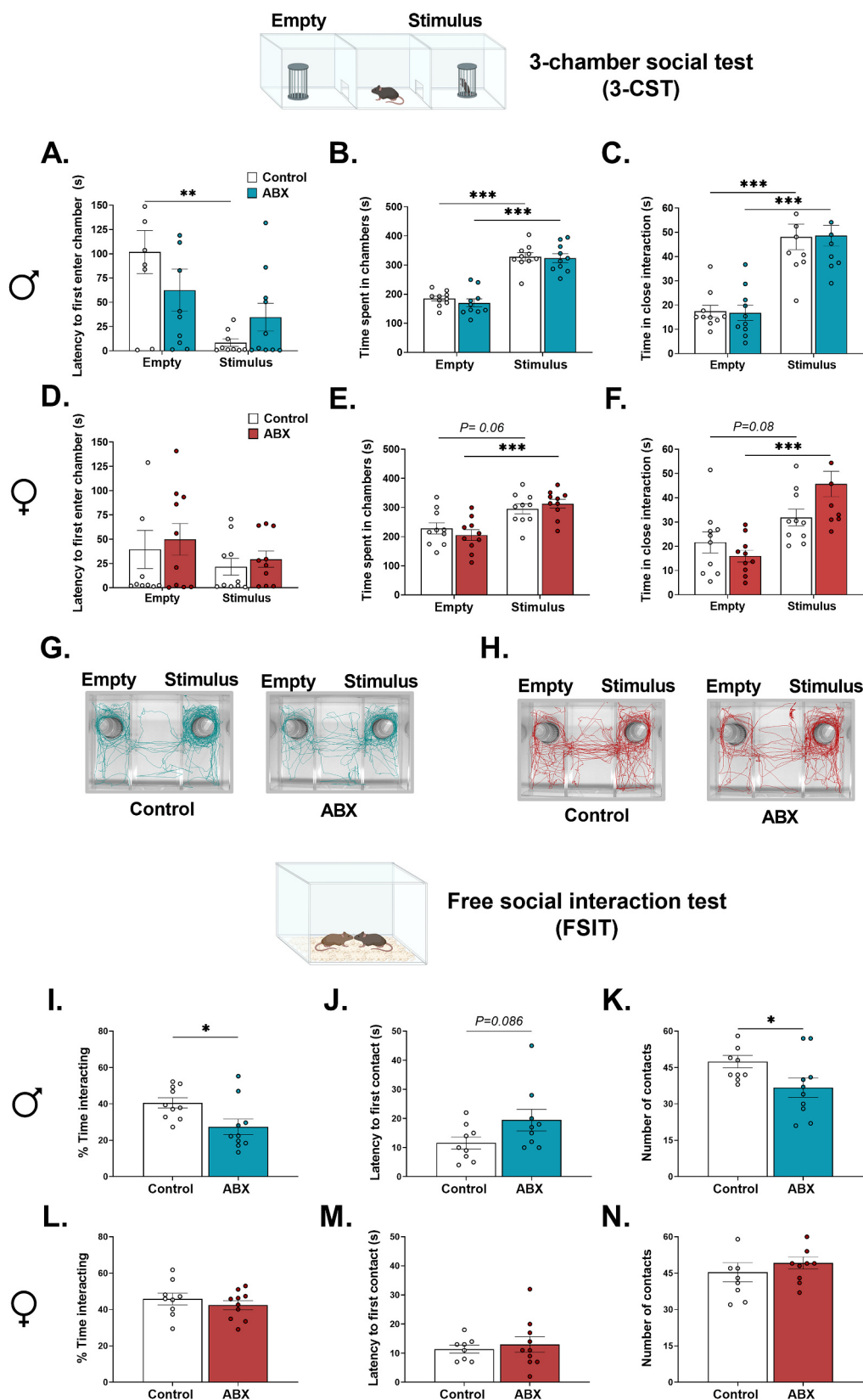


Fig. 3. Social behavior of juvenile offspring is affected by perinatal ABX exposure in a sex-specific manner. (A–H) Social behavior was evaluated by using a 3-chamber social approach task (3-CST) with the following parameters measured: (A,D) latency to first enter a chamber (in seconds), (B, E) time spent in each chamber (in seconds), (C, F) time spent in close interaction with either the stimulus mouse or the empty cage (in seconds) and (G, H) representative tracks of movement patterns of control and perinatally ABX-exposed male ($n = 8–10$ per group) and female ($n = 7–10$ per group) offspring. (I–N) Social behavior was also evaluated by using a free social interaction test (FSIT) with the following parameters measured: (I, L) percentage of time spent interacting with the stimulus mouse, (J, M) latency to first contact (in seconds) and (K, N) number of contacts with the stimulus mouse of control and perinatally ABX-exposed male ($n = 9–10$ per group) and female ($n = 9–10$ per group) juvenile offspring. All data are presented as means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to control offspring mice.

3.5. ABX-exposed male offspring display context-dependent alterations in anxiety-like behavior

To test for anxiety-like behaviors, we used the light-dark box (LDB; Fig. 4A–D), elevated-plus maze (EPM; Fig. 4E and F), and open-field (OF; Fig. 4G–L) tests. In the LDB test, perinatally ABX-exposed male juvenile offspring spent significantly more time in the light compartment than their respective control group (Treatment \times Compartment interaction: $[F_{(1, 36)} = 14.05, p = 0.0006]$; Bonferroni's *post hoc* comparison: control vs. ABX in the light compartment: $p = 0.0238$) (Fig. 4A). In females, however, there were no significant differences in time spent in the different compartments between control and perinatally ABX-exposed mice (Fig. 4B). Representative tracks of movement patterns of perinatally ABX-exposed male and female juvenile offspring during the 5-min LDB test are shown in Fig. 1C and D.

Results obtained in the EPM test were consistent with the data from the LDB test. Perinatally ABX-exposed male, but not female, juvenile offspring spent significantly less time in the closed arms compared to their respective controls (Treatment \times Maze Zone interaction: $[F_{(2, 51)} = 9.351, p = 0.0003]$; Bonferroni's *post hoc* comparison: control vs. ABX in closed arms: $p = 0.0027$) (Fig. 4E). No significant differences were observed in distance travelled in the EPM (data not shown).

In addition to the LDB and EPM tests, we also analyzed time spent and distance travelled in the center zone of the OF arena under low- or high-illumination. In the low-light OF test, perinatally ABX-exposed male juvenile offspring spent significantly less time ($p = 0.0392$) and travelled less distance ($p = 0.0419$) in the center of the OF arena (Fig. 4G, H, K). However, no significant differences were found in the case of the perinatally ABX-exposed female juvenile offspring (Fig. 4I, J, L). Interestingly, the perinatally ABX-exposed juvenile offspring displayed a different behavioral response under high-illumination. In males, no significant differences were observed between ABX-exposed and control groups (Figs. S2A and B). On the other hand, perinatally ABX-exposed female juvenile offspring spent more time in the center zone of the OF arena, but this trend did not reach statistical significance (Welch's *t*-test, $p = 0.0919$) (Figs. S2C and D).

3.6. Perinatal ABX exposure did not impair locomotor or exploratory activity of juvenile offspring

Locomotor and exploratory activity were assessed in the OF, LDB, and EPM tests, as measured by total distance travelled and number of rears, respectively (Fig. S3). In addition, we explored potential alterations in spatial memory and/or presence of repetitive behaviors, as measured by the pattern of visits to the four corners of the OF arena (Figs. S2G and H). However, we did not find any significant differences in any of the parameters investigated.

3.7. Perinatal ABX-exposed offspring show reduced oxytocin receptor gene expression in prefrontal cortex

To explore potential molecular mechanisms underlying the observed behavioral changes, we performed gene expression analysis of neuropeptides regulating social-emotional behavior, including oxytocin (*Oxt*) and corticotropin-releasing hormone (*Crh*), and their receptors (e.g., oxytocin receptor, *Oxtr*; Crh receptor 1, *Crhr1*). Neuropeptides mRNA levels were quantified in the hypothalamus (Fig. 5A–D), while gene expression of neuropeptide receptors was measured in the amygdala (Fig. 5E–H) and the prefrontal cortex (PFC) (Fig. 5I–L). Our results revealed that perinatal ABX exposure did not alter *Oxt* and *Crh* gene expression in the hypothalamus in the juvenile male and female offspring (Fig. 5A–D). Similarly, mRNA expression levels of the *Oxtr* and *Crhr1* were not impaired in the amygdala after ABX exposure, in juvenile offspring, regardless of sex (Fig. 5E–H). However, we found that expression levels of *Oxtr* in the PFC were significantly decreased following perinatal ABX exposure, more markedly in males ($p = 0.0085$)

(Fig. 5I) than in females ($p = 0.0491$) (Fig. 5K). On the other hand, *Crhr1* expression was similar between control and ABX-exposed male and female juvenile offspring (Fig. 5J, L). Concurrently, we examined the pattern of *Oxtr* and *Crhr1* expression and their cellular localization in the PFC by RNAscope *in situ* hybridization (Fig. 5M–Q). Our neuroanatomical investigation revealed a sparse expression pattern for *Oxtr* whereas *Crhr1* was strongly and homogeneously expressed in the PFC (Fig. 5M and N). Consistent with our RT-qPCR data, we showed that males and females offspring from ABX-treated dams displayed a reduced *Oxtr* gene expression compared to controls, whereas the distribution and expression of *Crhr1* mRNA was similar between the two groups (Fig. 5M and N). In addition, higher magnification photomicrographs revealed that *Oxtr* and *Crhr1* were co-expressed in a few PFC cells, in both males and females (Fig. 5P and Q).

In parallel, vasopressin (*Avp*) (which derives from the same precursor protein as oxytocin) and its receptor, the vasopressin receptor 1a (*Avpr1a*), were investigated for their gene expression in the same three brain regions. On one hand, no significant changes were found between ABX-exposed and control males in *Avp* or *Avpr1a* gene expression in neither of the brain regions studied (Figs. S4A, C, E). On the other hand, females from the perinatally ABX-exposed group did not reveal any difference in gene expression (Figs. S4B and F), except in the amygdala with a significant, though relatively variable, increase in *Avpr1a* gene expression in perinatally ABX-exposed females compared to matched controls ($p = 0.0510$) (Fig. S4D).

3.8. BBB integrity in the PFC is compromised in juvenile offspring from ABX-treated dams

To investigate the potential impact of perinatal ABX exposure on BBB integrity and brain inflammatory profile, we measured the gene expression of key tight junction proteins and cytokines in the PFC of juvenile offspring (Fig. S5). BBB integrity of perinatally ABX-exposed male offspring was significantly disrupted, as reflected by a significant reduction in the gene expression of *claudin-3* ($p = 0.0422$), *zonula occludens 1* (*ZO-1*, $p < 0.0001$) and *junctional adhesion molecule A* (*JAM-A*, $p = 0.0005$) (Fig. S5A). Juvenile female offspring from ABX-treated dams showed a similar decrease in *claudin-3* ($p = 0.0036$), *ZO-1* ($p = 0.0162$) and a tendency for a reduction in *ZO-1* and *JAM-A* gene expression ($p = 0.0803$) (Fig. S5B). However, we did not observe any significant changes in *IL-1 β* , and *IL-6* (cytokines with potent pro-inflammatory properties) gene expression in the PFC of perinatally ABX-exposed male and female juvenile offspring (Figs. S5C and D). On the other hand, the gene expression of *IL-10* (a cytokine with potent anti-inflammatory properties) was significantly reduced in the PFC of perinatally ABX-exposed female juvenile offspring ($p = 0.0058$), while juvenile males from ABX-exposed dams only showed a tendency for a reduction in its gene expression ($p = 0.0665$) (Figs. S5C and D).

3.9. Perinatal ABX-exposed juvenile male offspring show a mild inflammatory response in colon

Given that chronic inflammation of the gut can lead to behavioral changes, we examined the gene expression of the same tight junction proteins and cytokines in the colon of perinatally ABX-exposed juvenile offspring. We found no significant changes in the expression of tight junction proteins in the colon of perinatally ABX-exposed male and female juvenile offspring (Figs. S5E and F). However, the expression of the pro-inflammatory cytokine *IL-1 β* was significantly increased in the colon of male ($p = 0.0276$), but not female, juvenile offspring from ABX-treated dams (Figs. S5G–H).

4. Discussion

There is growing concern that commonly prescribed antibiotics during early life may have detrimental long-term effects on brain

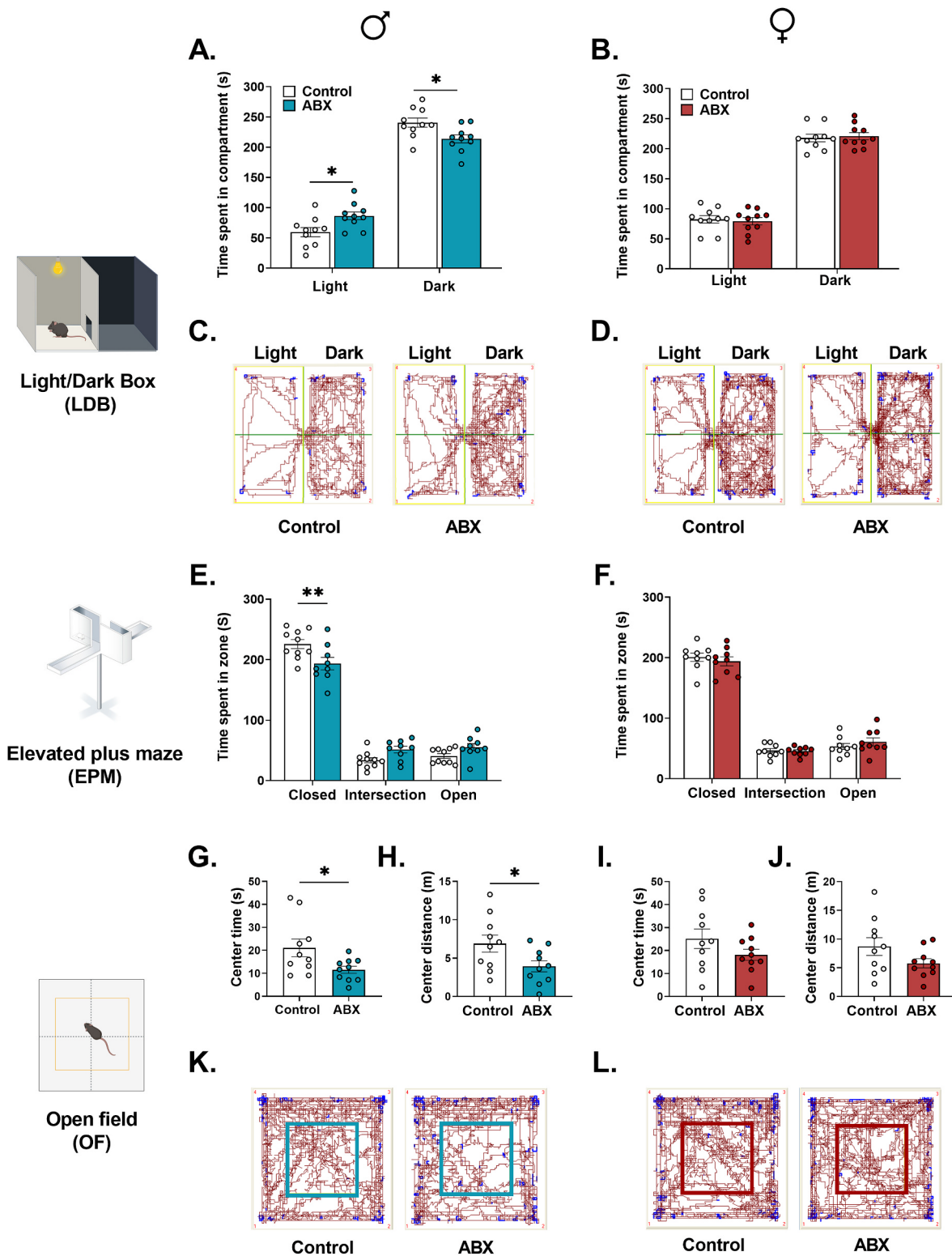


Fig. 4. Perinatal ABX exposure leads to context- and sex-dependent alterations in anxiety-like behavior, in juvenile offspring. (A–D) Anxiety-like behavior was first assessed by using the light-dark box (LDB) test, time spent in each compartment (in seconds) (A, B) and representative tracks of movement patterns (C, D) of control and perinatally ABX-exposed male ($n = 9-10$ per group) and female ($n = 9$ per group) juvenile offspring (E, F) in the elevated-plus maze (EPM), time spent in the three zones (in seconds) by control and perinatally ABX-exposed male ($n = 9-10$ per group) and female ($n = 9$ per group) juvenile offspring was evaluated. (G, L) In the open-field (OF) test, time spent, and distance travelled in the center zone of the arena (G–J), and representative tracks of movement patterns in the arena (K, L) of control and perinatally ABX-exposed male ($n = 9-10$ per group) and female ($n = 10$ per group) juvenile offspring were assessed. All data are presented as means \pm SEM. * $P < 0.05$ compared to control offspring mice.

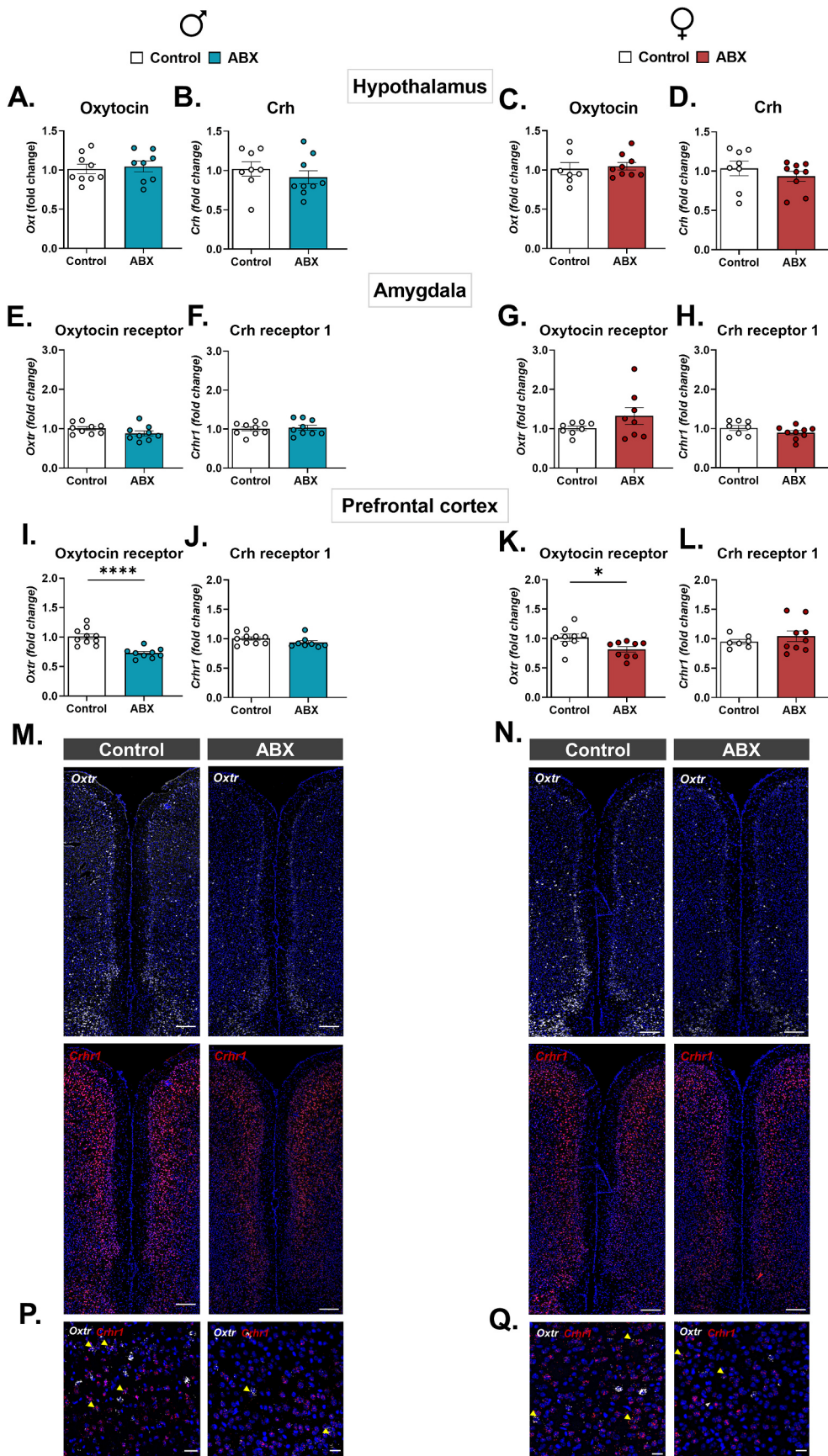


Fig. 5. Perinatal ABX exposure alters expression of socially-relevant neuropeptides and their receptors in the brain of offspring. (A–D) mRNA expression levels of oxytocin and corticotropin-releasing hormone (crh) in the hypothalamus of control and perinatally ABX-exposed male (n = 6 per group) and female (n = 5–6 per group) juvenile offspring. (E, K) mRNA expression levels of the oxytocin receptor (*Oxtr*) and the crh receptor 1 (*Crhr1*) in the amygdala (E–H) and prefrontal cortex (I–L) of control and perinatally ABX-exposed male (n = 8–10 per group) and female (n = 9 per group) juvenile offspring. (M, N) Representative photomicrographs of the medial prefrontal cortex (mPFC, 20X magnification) for *Oxtr* (white spots) and *Crhr1* (red spots) mRNA expression, as determined by RNAscope *in situ* hybridization, showing a decreased expression of the *Oxtr* in perinatally ABX-exposed animals vs controls, but no alteration in the distribution and expression of *Crhr1*. (P, Q) Photomicrographs of *Oxtr/Crhr1* double mRNA labelling (40X magnification) in the mPFC of control and ABX-exposed males (P) and females (Q), showing that few cells co-express *Oxtr* and *Crhr1* (yellow arrow-heads) in both males and females. All data (A–L) are presented as means ± SEM. *P < 0.05; **P < 0.01 compared to control offspring mice. Scale bars: (M, N) 200 µm; (P, Q) 25 µm.

development, altering neurodevelopmental trajectories and increasing susceptibility to mental health disorders in childhood and adulthood (Lavebratt et al., 2019; Slykerman et al., 2019; Tao et al., 2022; Otten et al., 2022). Here, we show that antibiotic-induced perturbation of the maternal microbiota during a narrow critical developmental window (last week of pregnancy and first three postnatal days) leads to changes in the microbiota composition of the juvenile offspring. Moreover, our results revealed sex-specific alterations in neonatal ultrasonic communication, social behavior, as well context-dependent anxiety-like behavior during the prepubertal period. In line with this behavioral phenotype, the gene expression of the oxytocin receptor (OXTR) and several tight-junction proteins were altered in the PFC of perinatally ABX-exposed juvenile offspring, which was more pronounced in the male offspring. Our findings indicate that antibiotic-induced perturbation of the maternal gut microbiota during late pregnancy and first few postpartum days influences offspring's early neurobehavioral outcomes in a sex-dependent manner and thus, advocating for more surveillance of infants exposed to ABX treatment in early-life.

Consistent with prior literature (Leclercq et al., 2017; McDonnell et al., 2021; Madany et al., 2022), our data demonstrate that exposure to a single broad-spectrum antibiotic (ampicillin) in early-life had enduring effects on the gut microbiota composition and diversity of juvenile offspring (*i.e.*, almost four weeks after antibiotic treatment was discontinued), without producing any major changes in body weight. Interestingly, we found an overrepresentation of Gram-negative bacteria such as Parabacteroides, Alistipes, Bacteroides and Akkermansia genera, in the gut microbiota of perinatally ABX-exposed juvenile offspring. These Gram-negative bacteria are a potential source of lipopolysaccharide (LPS; a major component of their outer membrane) that is thought to be one of the most potent activators of proinflammatory host responses. Fascinatingly, recent studies have revealed that the strong host proinflammatory responses that is commonly triggered by LPS-derived from pathogenic bacteria are, in fact, actively repressed by microbiota-derived LPS via silencing of Toll-like receptor 4 (TLR4) pathway (d'Hennessy et al., 2017). Although the significance of the immunoinhibitory activity of microbiota-derived LPS for gut-brain communication is yet to be understood, we have previously shown that TLR4 general knockout mice display reduced social interaction and increased anxiety-like behavior (Femenia et al., 2018), a phenotype that resembles in part, that of perinatally ABX-exposed male juvenile offspring. We therefore postulate that some of these bacterial changes observed in the gut microbiota of perinatally ABX-exposed offspring could be involved in the observed neurobehavioral alterations.

Mouse pups emit USVs when isolated from their dams and littermates to elicit a parental retrieval behavior, and thus can be considered an early form of communication that increases the pup's chances of survival within the nest (Grimsley et al., 2011; Wiaderkiewicz et al., 2013; Hofer et al., 2002). In the present study, we provide for the first time a complete description of the impact of perinatal ABX exposure on USV parameters and repertoire of both male and female pups. Our data show that the perinatally ABX-exposed neonatal offspring displayed an altered USV pattern, which was more pronounced in the male offspring. These alterations were particularly evident at P7 when body weight did not differ between control and perinatally ABX-exposed male offspring. Thus, perinatally ABX-exposed male pups vocalized more and longer USV calls compared to their respective control group at P7, but with lower frequency. Moreover, they showed changes in the structure of their vocal repertoire, producing more downward and step-up calls (the most abundant calls in our study) at both P3 and P7. Interestingly, this USV behavioral profile of perinatally ABX-exposed male pups resembles that of the inbred BTBR T + tf/J (BTBR) mouse strain, a commonly used animal model of core behavioral deficits in ASD (*i.e.*, repetitive behaviors and deficits in sociability and communication) at a comparable age (Scattoni et al., 2008).

Studies in GF mice have shown an influence of the gut microbiota on social behavior (Sherwin et al., 2019). Although some inconsistencies in

the direction of changes are apparent (Arentsen et al., 2015; Desbonnet et al., 2014), all studies highlight that absence of gut microbiota profoundly affects development of social behavior. Moreover, a recent study found that a prolonged exposure to a low-dose of penicillin, extending from last week of gestation and up to weaning at P21, resulted in reduced social behavior of adolescent offspring (Leclercq et al., 2017). In the present study, we found evidence for increased fear to novel social conditions in the juvenile male offspring from ABX-exposed dams, as reflected by their longer latency to first enter the chamber containing a novel mouse. After entering the chamber, however, both groups spent similar amount of time interacting with the novel stimulus mouse. In line with above observations, we found that juvenile male offspring from ABX-treated dams exhibited longer latency to initiate contact with a novel stimulus mouse in the FSIT. In addition, they had less contacts and interactions with the novel stimulus mouse. On the other hand, we found that juvenile female offspring from ABX-treated dams showed increased sociability in the 3-CST, while no evidence of altered social behavior in the FSIT. Overall, these results are consistent with both clinical and animal studies showing that males are more susceptible than females to perinatal insults such as preterm birth, stress, infection, and high-fat diet (for a review, see (Jaggard et al., 2020)).

One of the most reproducible behavioral traits of GF mice is decreased anxiety-like behavior (Clarke et al., 2013; Diaz Heijtz et al., 2011; Neufeld et al., 2011). Here, we found that juvenile male offspring from ABX-treated dams displayed decreased anxiety-like behavior in the LDB and EPM tests, whereas in the OF test they showed increased anxiety-like behavior, indicating a context-dependent anxiety-like behavior (*i.e.*, where the environment plays a key role). In consonance with these observations, certain types of environmental exposures (*e.g.*, parental loss, parenting style, abuse) during childhood have been associated with increased risk of developing anxiety disorders (Hettema and Ressler, 2015). Previous studies examining the impact of antibiotics across different developmental time windows have obtained variable results with respect to anxiety-like behavior. For instance, previous studies have shown that exposure to a cocktail of antibiotics from early weaning onwards (*i.e.*, P21–P80) (Desbonnet et al., 2015), leads to a decrease in anxiety-like behavior, while exposure only during adolescence (from P28 until P49) increases anxiety-like behavior (Lach et al., 2020). A recent study investigated the impact of a cocktail of antibiotics during three “putative critical windows” of postnatal development (*i.e.*, early postnatal: P2-9, pre-weaning: P12-18 or post-weaning: P21-27) on neuroimmune function and behavior (Lynch et al., 2023). The authors found sex and time-dependent effects of this treatment on anxiety-related behavioral outcomes in adolescence and adulthood, with female mice exposed during the pre-weaning period exhibiting pronounced anxiety-like behavior (Lynch et al., 2023). Intriguingly, we did not find any differences in anxiety-like behavior in the juvenile female offspring from ABX-exposed dams. Others have also found differential effects of perinatal antibiotic exposure on male and female offspring. Exposure to a low-dose penicillin from last week of pregnancy and up to weaning (P21) decreases anxiety-like behavior in the adolescent male, but not female offspring (Leclercq et al., 2017). In contrast, exposure to the same antibiotic regimen during the last week of pregnancy leads to a decrease in anxiety-like behavior only in adolescent female offspring (Champagne-Jorgensen et al., 2020). Taken together, the above findings indicate that the effects of early-life antibiotic exposure on anxiety-like behavior seems to be highly dependent upon factors such as the type of antibiotic, dose regimen (single vs. cocktail), and developmental time window when such exposure occurs.

We have previously shown that GF mice display increased novelty-induced motor activity (*i.e.*, locomotor and rearing activity) in the OF test ((Diaz Heijtz et al., 2011; Arentsen et al., 2015), but see (Neufeld et al., 2011)), a finding consistent with their elevated home-cage activity (Backhed et al., 2007). In this study, we did not find evidence that perturbation of the maternal gut microbiota, during E13 to P3, alters novelty-induced motor activity of juvenile offspring. In contrast to our

findings, Tochitani and colleagues showed that exposure to a cocktail of non-absorbable antibiotics during mid pregnancy (E9-E16) in C57BL/6J mice leads to a decrease in locomotor activity of juvenile (P28–P32), but not adult, offspring in both familiar and novel environments (Tochitani et al., 2016). It is worthwhile mentioning that the timing of antibiotic exposure in their study overlapped with the neurogenesis of murine midbrain dopamine neurons (*i.e.*, beginning at E9.5, peaking at E11.5 and then ceasing at around E14.5–E15.5) (Asgrimsdottir and Arenas, 2020), suggesting a potential delay in the maturation of the midbrain DA system, critical for proper motor control.

Oxytocin (Oxt), arginine-vasopressin (Avp) and corticotropin-releasing hormone (Crh) are important modulators of social and affective behaviors, including mother-pup attachment in early life and later sociability, as well as stress coping and anxiety (Caldwell, 2017; Kompier et al., 2019; Li et al., 2016). Given the observed changes in social and anxiety-like behaviors, especially in male offspring from ABX-treated dams, we explored whether the expression of these neuropeptides and their receptors in relevant brain regions might be altered. Our quantification of Oxt or Avp gene expression in the hypothalamus of juvenile offspring following ABX exposure revealed no significant changes as compared to controls. Our findings do not correlate with previous studies reporting that an ABX treatment resulted in a significant decrease (Desbonnet et al., 2015) or an increase of Avp gene expression (Johnson and Burnet, 2020). We also found that hypothalamic Crh levels were similar between ABX-exposed and control juveniles, which is consistent to what has been reported in the literature (Desbonnet et al., 2015; Wu et al., 2021). Concurrently, we found a significant decrease of Oxt gene expression in the PFC of male offspring from ABX-exposed dams, that positively correlates with the social behavioral outcomes observed (*i.e.*, a lower social motivation). Our findings are in agreement with a previous study reporting that exposure to an ABX cocktail during adolescence led to a similar reduction in Oxt gene expression in the frontal cortex of males (Johnson and Burnet, 2020). By contrast, other authors found that administration of low-dose penicillin in the last week of pregnancy (Champagne-Jorgensen et al., 2020), or during the postnatal period (*i.e.*, from P14 to P21) (Kayyal et al., 2020), did not impair Oxt gene expression in the PFC, whereas alterations in sociability were observed. Our data also revealed that gene expression of Avpr1a was not modified by the ABX treatment in the hypothalamus, whereas it was increased in the amygdala, a key node of the social behavior network, but only in females. This supports the idea that the neuropeptidic systems regulate social and affective behaviors in a sex-specific manner (Nakajima et al., 2014; Bredewold and Veenema, 2018; Resendez et al., 2020).

Altogether, our data reveal that antibiotic-induced perturbation of the maternal gut microbiota during the perinatal period impact the development of the Oxt neuropeptidic system in the PFC, that may contribute to the alterations in social and emotional behaviors, especially observed in males. Interestingly, it was recently shown that a small population of Oxt-responsive interneurons in the PFC modulate social and anxiety-like behaviors in a sex-dependent manner (Li et al., 2016). Specifically, these Oxt-responsive interneurons promote sociosexual behavior in female, but not male mice. On the other hand, they attenuate anxiety-like behavior in male, but not female mice (Li et al., 2016). In future studies, it would be important to explore whether this population of Oxt-responsive interneurons are particularly vulnerable to antibiotic exposure in early-life, and thus contributing to atypical social and emotional development, as observed in the present study.

The functional and structural integrity of the BBB is pivotal for maintaining homeostasis of the brain microenvironment and preventing unwanted toxins and pathogens from entering the brain (Obermeier et al., 2013). Previous studies have shown that commensal gut bacteria regulate the expression of tight junction proteins such as occludin and claudin-5 in endothelial cells that are mainly responsible for barrier properties, and thus BBB permeability (Braniste et al., 2014). In our study, ABX exposure started at E13 and continued until the first three

postnatal days, which overlaps with the developmental time window of BBB formation. Fascinatingly, we found that similar to GF mice, perinatally ABX-exposed juvenile offspring showed a reduction in several key tight junction proteins (*i.e.*, occludin, claudin-3, ZO-1, JAM-A) in the PFC, particularly in the male offspring, indicating a compromised BBB integrity. Multiple studies have highlighted the regulation of the tight junction barrier by inflammatory cytokines (for a review, see (Capaldo and Nusrat, 2009)). Surprisingly, we did not observe any changes in the expression of classical pro-inflammatory cytokines (IL-1 β , IL-6) in the PFC, as had been seen previously after a prolonged exposure to a low-dose of penicillin (Leclercq et al., 2017) or after a cocktail of antibiotics during late gestation through birth (Madany et al., 2022). The observed abnormalities in the regulation of tight junction proteins in the PFC are interesting (regardless of the mechanisms involved) and suggest that an altered BBB may contribute to the behavioral changes observed in the perinatally ABX-exposed juvenile offspring. Chen and colleagues demonstrated that exposure to a cocktail of antibiotics during pregnancy (E15 to delivery) alters the intestinal microbiota, increases intestinal inflammation, and disrupts the intestinal barrier in neonatal mice (Chen et al., 2021). In the present study, we did not observe any changes in the expression of tight junction proteins in the colon of perinatally ABX-exposed male and female offspring. However, we found a significant increase in IL-1 β in the colon of perinatally ABX-exposed male, but not female, offspring, indicating a mild inflammatory response. Thus, possibly contributing to the observed behavioral changes.

Previous work from our laboratory have demonstrated that exposure to a single-broad spectrum antibiotic (ampicillin) during the entire pregnancy reduces the taxonomic richness and diversity of the maternal gut microbiota, without affecting the body weight of the dams or offspring (Arentsen, 2017). Although we did not explore the molecular mechanisms underlying perinatal ABX-induced neurobehavioral changes observed in this study, new research points to the involvement of maternal gut microbiota-derived metabolites. Vuong and colleagues demonstrated that during pregnancy, the maternal microbiome regulates metabolites, not only in the maternal serum, but also in the brains of the fetal offspring (Vuong et al., 2020). The authors showed compelling evidence that specific fetal brain metabolites (*e.g.*, trimethylamine-*N*-oxide, 5-aminovalerate, imidazole propionate and hippurate), which are regulated by prenatal antibiotic exposure or GF condition, can promote fetal thalamocortical axogenesis. Other studies have shown that antibiotic can reduce the concentration of short chain fatty acids (SCFAs; primarily acetate, propionate, and butyrate, as end products), the main metabolites produced in the colon by bacterial fermentation of dietary fibers and resistant starch. SCFAs are crucial for maintaining intestinal homeostasis and act as key mediators of gut-brain communication (O’Riordan et al., 2022).

In conclusion, we found that perturbation of the maternal gut microbiota, induced by exposure to a single broad-spectrum antibiotic (ampicillin) during a narrow critical window of development, has sex-specific effects on ultrasonic communication, social and anxiety-like behavior in the neonatal-prepubertal mouse offspring. These behavioral changes were accompanied by changes in the gut microbiota composition and gene expression. In future studies it will be important to identify specific maternal gut microbiota-derived metabolites or bacterial components that might be associated with atypical neurodevelopment of offspring, as well as the molecular pathways underlying the higher vulnerability of the developing male brain to ABX exposure.

Author contributions

CM and RDH designed the study and wrote the manuscript. CM and IMS performed and analyzed the gene expression profiling, CM and RDH performed and analyzed the behavioral experiments. CM, YC and NC performed and analyzed the RNAscope experiments. IMS and NC revised and approved the final version of the manuscript.

Data availability

Data will be made available on request.

Acknowledgments

CM is the recipient of a scholarship co-funded by the Normandie Regional Council and Karolinska Institutet. This work was supported by the Swedish Research Council (2018–06232), Swedish Brain Foundation (FO2020-0088, FO2022-0199), Foundation Freemasons-Children's house in Stockholm and Institut National de la Santé et de la Recherche Médicale (INSERM U1239; NC) and the University of Rouen (NC).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuropharm.2023.109479>.

REFERENCES

- Antunes, M., Biala, G., 2012. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognit. Process.* 13 (2), 93–110.
- Arentsen, T., 2017. Gut Microbes and the Developing Brain. Karolinska Institutet, Stockholm.
- Arentsen, T., Raith, H., Qian, Y., Forssberg, H., Diaz Heijtz, R., 2015. Host microbiota modulates development of social preference in mice. *Microb. Ecol. Health Dis.* 26, 29719.
- Arentsen, T., Qian, Y., Gkotsis, S., Femenia, T., Wang, T., Udekwu, K., et al., 2017. The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior. *Mol. Psychiatr.* 22 (2), 257–266.
- Asgrimsdottir, E.S., Arenas, E., 2020. Midbrain dopaminergic neuron development at the single cell level: in vivo and in stem cells. *Front. Cell Dev. Biol.* 8, 463.
- Backhed, F., Manchester, J.K., Semenkovich, C.F., Gordon, J.I., 2007. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc. Natl. Acad. Sci. U. S. A.* 104 (3), 979–984.
- Bell, M.R., 2018. Comparing postnatal development of gonadal hormones and associated social behaviors in rats, mice, and humans. *Endocrinology* 159 (7), 2596–2613.
- Borre, Y.E., O'Keefe, G.W., Clarke, G., Stanton, C., Dinan, T.G., Cryan, J.F., 2014. Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol. Med.* 20 (9), 509–518.
- Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Toth, M., et al., 2014. The gut microbiota influences blood-brain barrier permeability in mice. *Sci. Transl. Med.* 6 (263), 263ra158.
- Bredewold, R., Veenema, A.H., 2018. Sex differences in the regulation of social and anxiety-related behaviors: insights from vasopressin and oxytocin brain systems. *Curr. Opin. Neurobiol.* 49, 132–140.
- Buffington, S.A., Di Prisco, G.V., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., Costa-Mattioli, M., 2016. Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. *Cell* 165 (7), 1762–1775.
- Buffington, S.A., Dooling, S.W., Sgritta, M., Noecker, C., Murillo, O.D., Felice, D.F., et al., 2021. Dissecting the contribution of host genetics and the microbiome in complex behaviors. *Cell* 184 (7), 1740–1756 e16.
- Caldwell, H.K., 2017. Oxytocin and vasopressin: powerful regulators of social behavior. *Neuroscientist* 23 (5), 517–528.
- Caldwell, H.K., Albers, H.E., 2016. Oxytocin, vasopressin, and the motivational forces that drive social behaviors. *Curr. Top Behav. Neurosci.* 27, 51–103.
- Capaldo, C.T., Nusrat, A., 2009. Cytokine regulation of tight junctions. *Biochim. Biophys. Acta* 1788 (4), 864–871.
- Caruso, A., Ricceri, L., Scattoni, M.L., 2020. Ultrasonic vocalizations as a fundamental tool for early and adult behavioral phenotyping of Autism Spectrum Disorder rodent models. *Neurosci. Biobehav. Rev.* 116, 31–43.
- Champagne-Jorgensen, K., Mian, M.F., Kay, S., Hanani, H., Ziv, O., McVey Neufeld, K.A., et al., 2020. Prenatal low-dose penicillin results in long-term sex-specific changes to murine behaviour, immune regulation, and gut microbiota. *Brain Behav. Immun.* 84, 154–163.
- Chen, C.M., Chou, H.C., Yang, Y.S.H., 2021. Maternal antibiotic treatment disrupts the intestinal microbiota and intestinal development in neonatal mice. *Front. Microbiol.* 12, 684233.
- Chini, M., Hanganu-Opat, I.L., 2021. Prefrontal cortex development in health and disease: lessons from rodents and humans. *Trends Neurosci.* 44 (3), 227–240.
- Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R.D., Shanahan, F., et al., 2013. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol. Psychiatr.* 18 (6), 666–673.
- Codagnone, M.G., Spichak, S., O'Mahony, S.M., O'Leary, O.F., Clarke, G., Stanton, C., et al., 2019. Programming bugs: microbiota and the developmental origins of brain health and disease. *Biol. Psychiatr.* 85 (2), 150–163.
- Cohen-Cory, S., Kidane, A.H., Shirkey, N.J., Marshak, S., 2010. Brain-derived neurotrophic factor and the development of structural neuronal connectivity. *Dev. Neurobiol.* 70 (5), 271–288.
- Cong, X., Xu, W., Romisher, R., Poveda, S., Forte, S., Starkweather, A., et al., 2016. Gut microbiome and infant health: brain-gut-microbiota Axis and host genetic factors. *Yale J. Biol. Med.* 89 (3), 299–308.
- Cowan, C.S.M., Dinan, T.G., Cryan, J.F., 2020. Annual Research Review: critical windows - the microbiota-gut-brain axis in neurocognitive development. *JCPP (J. Child Psychol. Psychiatry)* 61 (3), 353–371.
- Cox, L.M., Blaser, M.J., 2015. Antibiotics in early life and obesity. *Nat. Rev. Endocrinol.* 11 (3), 182–190.
- Cryan, J.F., O'Riordan, K.J., Cowan, C.S.M., Sandhu, K.V., Bastiaansen, T.F.S., Boehme, M., et al., 2019. The microbiota-gut-brain Axis. *Physiol. Rev.* 99 (4), 1877–2013.
- D'Amato, F.R., Scalera, E., Sarli, C., Moles, A., 2005. Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? *Behav. Genet.* 35 (1), 103–112.
- d'Hennezel, E., Abubucker, S., Murphy, L.O., Cullen, T.W., 2017. Total lipopolysaccharide from the human gut microbiome silences toll-like receptor signaling. *mSystems* 2 (6).
- Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T.G., Cryan, J.F., 2014. Microbiota is essential for social development in the mouse. *Mol. Psychiatr.* 19 (2), 146–148.
- Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R.D., et al., 2015. Gut microbiota depletion from early adolescence in mice: implications for brain and behaviour. *Brain Behav. Immun.* 48, 165–173.
- Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., et al., 2011. Normal gut microbiota modulates brain development and behavior. *Proc. Natl. Acad. Sci. U. S. A.* 108 (7), 3047–3052.
- Eagleson, K.L., Xie, Z., Levitt, P., 2017. The pleiotropic MET receptor network: circuit development and the neural-medical interface of autism. *Biol. Psychiatr.* 81 (5), 424–433.
- Erny, D., Hrabec de Angelis, A.L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., et al., 2015. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat. Neurosci.* 18 (7), 965–977.
- Fairless, A.H., Dow, H.C., Kreibich, A.S., Torre, M., Kuruvilla, M., Gordon, E., et al., 2012. Sociability and brain development in BALB/cJ and C57BL/6J mice. *Behav. Brain Res.* 228 (2), 299–310.
- Femenia, T., Qian, Y., Arentsen, T., Forssberg, H., Diaz Heijtz, R., 2018. Toll-like receptor-4 regulates anxiety-like behavior and DARPP-32 phosphorylation. *Brain Behav. Immun.* 69, 273–282.
- Firestein, M.R., Myers, M.M., Austin, J., Stark, R.I., Barone, J.L., Ludwig, R.J., et al., 2019. Perinatal antibiotics alter preterm infant EEG and neurobehavior in the Family Nurture Intervention trial. *Dev. Psychobiol.* 61 (5), 661–669.
- Fonseca, A.H., Santana, G.M., Bosque Ortiz, G.M., Bampi, S., Dietrich, M.O., 2021. Analysis of ultrasonic vocalizations from mice using computer vision and machine learning. *Elife* 10.
- Grimsley, J.M., Monaghan, J.J., Wenstrup, J.J., 2011. Development of social vocalizations in mice. *PLoS One* 6 (3), e17460.
- Guastella, A.J., Einfeld, S.L., Gray, K.M., Rinehart, N.J., Tonge, B.J., Lambert, T.J., et al., 2010. Intranasal oxytocin improves emotion recognition for youth with autism spectrum disorders. *Biol. Psychiatr.* 67 (7), 692–694.
- Gur, T.L., Palkar, A.V., Rajasekera, T., Allen, J., Niraula, A., Godbout, J., et al., 2019. Prenatal stress disrupts social behavior, cortical neurobiology and commensal microbes in adult male offspring. *Behav. Brain Res.* 359, 886–894.
- Hettema, J.M., 2015. The etiology of fear and anxiety: the role of environmental exposures. In: Ressler, Kerry J. (Ed.), *DSPE, Barbara Olasov Rothbaum, Anxiety Disorders*. Oxford University Press, Oxford, pp. 189–200.
- Hoban, A.E., Stilling, R.M., Ryan, F.J., Shanahan, F., Dinan, T.G., Claesson, M.J., et al., 2016. Regulation of prefrontal cortex myelination by the microbiota. *Transl. Psychiatr.* 6 (4), e774.
- Hofer, M.A., Shair, H.N., Brunelli, S.A., 2002. Ultrasonic vocalizations in rat and mouse pups. *Curr. Protoc. Neurosci.* (Chapter 8):Unit 8 14.
- Hornberg, H., Perez-Garci, E., Schreiner, D., Hatstatt-Burkle, L., Magara, F., Baudouin, S., et al., 2020. Rescue of oxytocin response and social behaviour in a mouse model of autism. *Nature* 584 (7820), 252–256.
- Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., et al., 2013. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155 (7), 1451–1463.
- Jaggard, M., Rea, K., Spichak, S., Dinan, T.G., Cryan, J.F., 2020. You've got male: sex and the microbiota-gut-brain axis across the lifespan. *Front. Neuroendocrinol.* 56, 100815.
- Johnson, K.V.A., Burnet, P.W.J., 2020. Opposing effects of antibiotics and germ-free status on neuropeptide systems involved in social behaviour and pain regulation. *BMC Neurosci.* 21 (1), 32.
- Kayyal, M., Javkar, T., Firoz Mian, M., Binyamin, D., Koren, O., McVey Neufeld, K.A., et al., 2020. Sex dependent effects of post-natal penicillin on brain, behavior and immune regulation are prevented by concurrent probiotic treatment. *Sci. Rep.* 10 (1), 10318.
- Kelsey, C.M., Prescott, S., McCulloch, J.A., Trinchieri, G., Valladares, T.L., Dreisbach, C., et al., 2021. Gut microbiota composition is associated with newborn functional brain connectivity and behavioral temperament. *Brain Behav. Immun.* 91, 472–486.
- Keogh, C.E., Kim, D.H.J., Pusceddu, M.M., Knotts, T.A., Rabasa, G., Sladek, J.A., et al., 2021. Myelin as a regulator of development of the microbiota-gut-brain axis. *Brain Behav. Immun.* 91, 437–450.
- Kim, S., Kim, H., Yim, Y.S., Ha, S., Atarashi, K., Tan, T.G., et al., 2017. Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring. *Nature* 549 (7673), 528–532.

- Kompier, N.F., Keyzers, C., Gazzola, V., Lucassen, P.J., Krugers, H.J., 2019. Early life adversity and adult social behavior: focus on arginine vasopressin and oxytocin as potential mediators. *Front. Behav. Neurosci.* 13, 143.
- Lach, G., Fulling, C., Bastiaanssen, T.F.S., Fouhy, F., Donovan, A.N.O., Ventura-Silva, A.P., et al., 2020. Enduring neurobehavioral effects induced by microbiota depletion during the adolescent period. *Transl. Psychiatry* 10 (1), 382.
- Lafforgue, G., Arellano, C., Vachoux, C., Woodley, J., Philibert, C., Dupouy, V., et al., 2008. Oral absorption of ampicillin: role of paracellular route vs. PePT1 transporter. *Fundam. Clin. Pharmacol.* 22 (2), 189–201.
- Lavebratt, C., Yang, L.L., Giacobini, M., Forsell, Y., Schalling, M., Partonen, T., et al., 2019. Early exposure to antibiotic drugs and risk for psychiatric disorders: a population-based study. *Transl. Psychiatry* 9 (1), 317.
- Leclercq, S., Mian, F.M., Stanisz, A.M., Bindels, L.B., Cambier, E., Ben-Amram, H., et al., 2017. Low-dose penicillin in early life induces long-term changes in murine gut microbiota, brain cytokines and behavior. *Nat. Commun.* 8, 15062.
- Ledger, W.J., Blaser, M.J., 2013. Are we using too many antibiotics during pregnancy? *BJOG* 120 (12), 1450–1452.
- Li, K., Nakajima, M., Ibanez-Tallon, I., Heintz, N., 2016. A cortical circuit for sexually dimorphic oxytocin-dependent anxiety behaviors. *Cell* 167 (1), 60–72 e11.
- Lord, C., Brugha, T.S., Charman, T., Cusack, J., Dumas, G., Frazier, T., et al., 2020. Autism spectrum disorder. *Nat. Rev. Dis. Prim.* 6 (1), 5.
- Lynch, C.M.K., Cowan, C.S.M., Bastiaanssen, T.F.S., Moloney, G.M., Theune, N., van de Wouw, M., et al., 2023. Critical windows of early-life microbiota disruption on behaviour, neuroimmune function, and neurodevelopment. *Brain Behav. Immun.* 108, 309–327.
- Madany, A.M., Hughes, H.K., Ashwood, P., 2022. Antibiotic treatment during pregnancy alters offspring gut microbiota in a sex-dependent manner. *Biomedicines* 10 (5).
- Maestro, S., Muratori, F., Cesari, A., Cavallaro, M.C., Paziante, A., Pecini, C., et al., 2005. Course of autism signs in the first year of life. *Psychopathology* 38 (1), 26–31.
- Marra, F., Marra, C.A., Richardson, K., Lynd, L.D., Kozyrskyj, A., Patrick, D.M., et al., 2009. Antibiotic use in children is associated with increased risk of asthma. *Pediatrics* 123 (3), 1003–1010.
- McDonnell, L., Gilkes, A., Ashworth, M., Rowland, V., Harries, T.H., Armstrong, D., et al., 2021. Association between antibiotics and gut microbiome dysbiosis in children: systematic review and meta-analysis. *Gut Microb.* 13 (1), 1–18.
- Meyer-Lindenberg, A., Domes, G., Kirsch, P., Heinrichs, M., 2011. Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. *Nat. Rev. Neurosci.* 12 (9), 524–538.
- Nakajima, M., Gorlich, A., Heintz, N., 2014. Oxytocin modulates female sociosexual behavior through a specific class of prefrontal cortical interneurons. *Cell* 159 (2), 295–305.
- Neufeld, K.M., Kang, N., Bienenstock, J., Foster, J.A., 2011. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neuro Gastroenterol. Motil.* 23 (3), 255–264, e119.
- O'Connor, R., Moloney, G.M., Fulling, C., O'Riordan, K.J., Fitzgerald, P., Bastiaanssen, T.F.S., et al., 2021. Maternal antibiotic administration during a critical developmental window has enduring neurobehavioural effects in offspring mice. *Behav. Brain Res.* 404, 113156.
- O'Riordan, K.J., Collins, M.K., Moloney, G.M., Knox, E.G., Aburto, M.R., Fulling, C., et al., 2022. Short chain fatty acids: microbial metabolites for gut-brain axis signalling. *Mol. Cell. Endocrinol.* 546, 111572.
- Obermeier, B., Daneman, R., Ransohoff, R.M., 2013. Development, maintenance and disruption of the blood-brain barrier. *Nat. Med.* 19 (12), 1584–1596.
- Otten, K., Keller, L., Puiui, A.A., Herpertz-Dahlmann, B., Seitz, J., Kohn, N., et al., 2022. Pre- and postnatal antibiotic exposure and risk of developing attention deficit hyperactivity disorder-A systematic review and meta-analysis combining evidence from human and animal studies. *Neurosci. Biobehav. Rev.* 140, 104776.
- Panksepp, J.B., Lahvis, G.P., 2007. Social reward among juvenile mice. *Gene Brain Behav.* 6 (7), 661–671.
- Resendez, S.L., Namboodiri, V.M.K., Otis, J.M., Eckman, L.E.H., Rodriguez-Romaguera, J., Ung, R.L., et al., 2020. Social stimuli induce activation of oxytocin neurons within the paraventricular nucleus of the hypothalamus to promote social behavior in male mice. *J. Neurosci.* 40 (11), 2282–2295.
- Rincel, M., Aubert, P., Chevalier, J., Grohard, P.A., Basso, L., Monchoux de Oliveira, C., et al., 2019. Multi-hit early life adversity affects gut microbiota, brain and behavior in a sex-dependent manner. *Brain Behav. Immun.* 80, 179–192.
- Scattoni, M.L., Gandhi, S.U., Ricceri, L., Crawley, J.N., 2008. Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PLoS One* 3 (8), e3067.
- Scattoni, M.L., Crawley, J., Ricceri, L., 2009. Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. *Neurosci. Biobehav. Rev.* 33 (4), 508–515.
- Semple, B.D., Blomgren, K., Gimlin, K., Ferrero, D.M., Noble-Haeusslein, L.J., 2013. Brain development in rodents and humans: identifying benchmarks of maturation and vulnerability to injury across species. *Prog. Neurobiol.* 106–107, 1–16.
- Sherwin, E., Bordenstein, S.R., Quinn, J.L., Dinan, T.G., Cryan, J.F., 2019. Microbiota and the social brain. *Science* 366 (6465).
- Slykerman, R.F., Coomarasamy, C., Wickens, K., Thompson, J.M.D., Stanley, T.V., Barthow, C., et al., 2019. Exposure to antibiotics in the first 24 months of life and neurocognitive outcomes at 11 years of age. *Psychopharmacology (Berl)* 236 (5), 1573–1582.
- Tao, Q., Shen, Y., Li, Y., Luo, H., Yuan, M., Gan, J., 2022. Prenatal exposure to antibiotics and risk of neurodevelopmental disorders in offspring: a systematic review and meta-analysis. *Front. Neurol.* 13, 1045865.
- Thion, M.S., Low, D., Silvin, A., Chen, J., Grisel, P., Schulte-Schrepping, J., et al., 2018. Microbiome influences prenatal and adult microglia in a sex-specific manner. *Cell* 172 (3), 500–516 e16.
- Tochitani, S., Ikeno, T., Ito, T., Sakurai, A., Yamauchi, T., Matsuzaki, H., 2016. Administration of non-absorbable antibiotics to pregnant mice to perturb the maternal gut microbiota is associated with alterations in offspring behavior. *PLoS One* 11 (1), e0138293.
- Volkova, A., Ruggles, K., Schulfer, A., Gao, Z., Ginsberg, S.D., Blaser, M.J., 2021. Effects of early-life penicillin exposure on the gut microbiome and frontal cortex and amygdala gene expression. *iScience* 24 (7), 102797.
- Vuong, H.E., Pronovost, G.N., Williams, D.W., Coley, E.J.L., Siegler, E.L., Qiu, A., et al., 2020. The maternal microbiome modulates fetal neurodevelopment in mice. *Nature* 586 (7828), 281–286.
- Wiaderkiewicz, J., Glowacka, M., Grabowska, M., Jaroslaw-Jerzy, B., 2013. Ultrasonic vocalizations (USV) in the three standard laboratory mouse strains: developmental analysis. *Acta Neurobiol. Exp.* 73 (4), 557–563.
- Wu, W.L., Adame, M.D., Liou, C.W., Barlow, J.T., Lai, T.T., Sharon, G., et al., 2021. Microbiota regulate social behaviour via stress response neurons in the brain. *Nature* 595 (7867), 409–414.
- Zhang, L., Wang, Y., Xiayu, X., Shi, C., Chen, W., Song, N., et al., 2017. Altered gut microbiota in a mouse model of alzheimer's disease. *J. Alzheimers Dis.* 60 (4), 1241–1257.

Results – Publication: Supplementary data

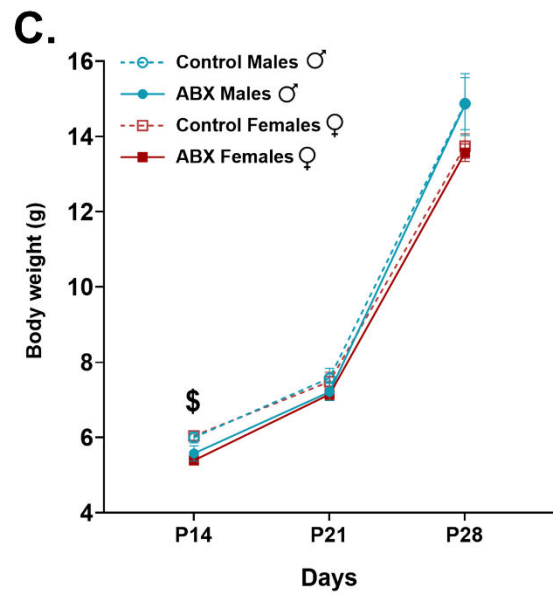
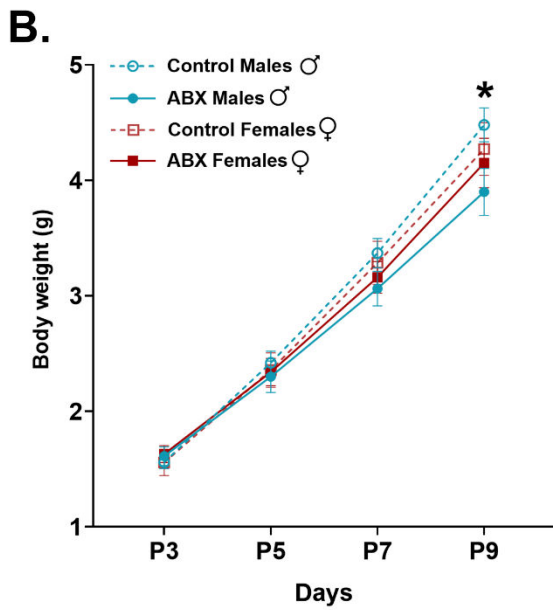
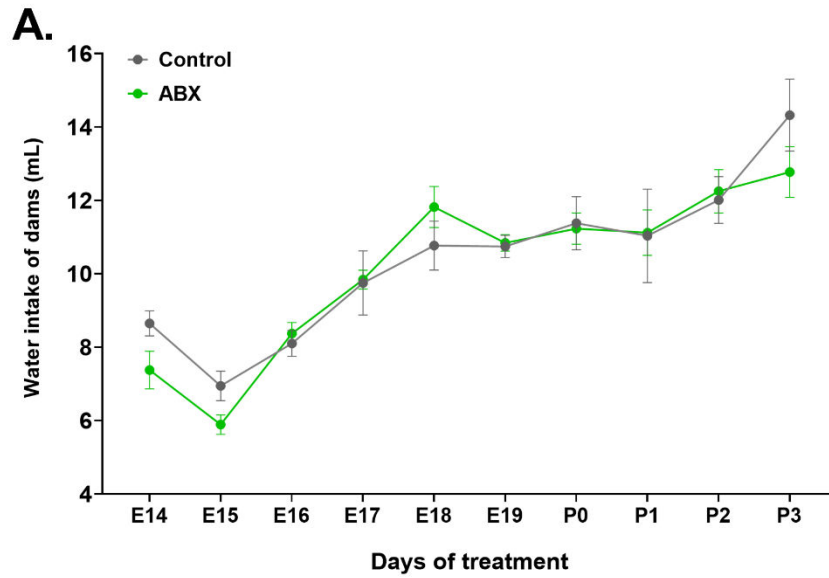
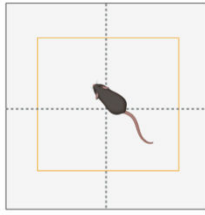


Figure S1. Perinatal ABX exposure does not affect dam water intake or offspring body weight. (A) Daily water intake from E14 to P3 of control (n = 9) and ABX-treated dams (n = 10). (B, C) Body weight of male (blue) and female (red) offspring in early postnatal life (B) (P3 to P9) of control and perinatally ABX-exposed male (n = 8–10 per group) and female (n = 7–10 per group) offspring and during the juvenile period (C) (P14 to P28; males: n = 9–14 per group and females: n = 8–17 per group). All data are presented as means \pm SEM. *P < 0.05: perinatally ABX-exposed males vs control males at P9. \$P < 0.05: Perinatally ABX-exposed females vs control females. associated with the disruption of the blood brain barrier (BBB) and a significant reduction in the oxytocin receptor (OXTR) in the prefrontal cortex (PFC).



High-light
Open field (HOF)

♂

♀

□ Control ■ ABX

□ Control ■ ABX

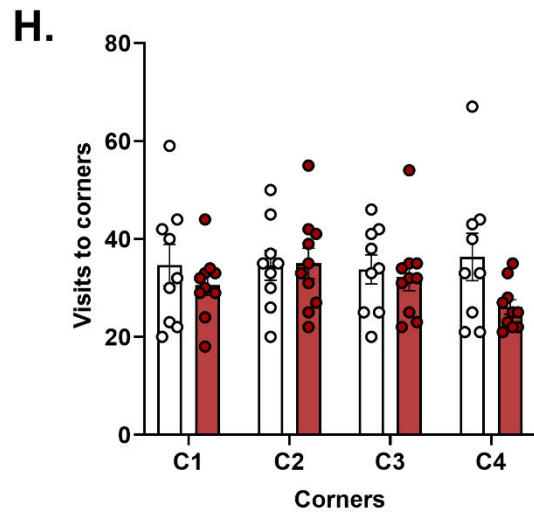
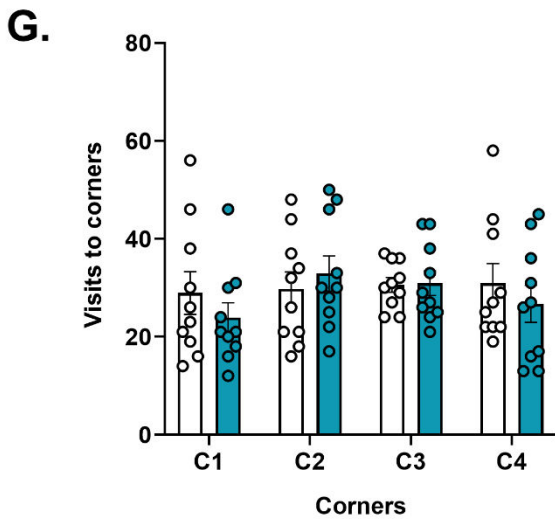
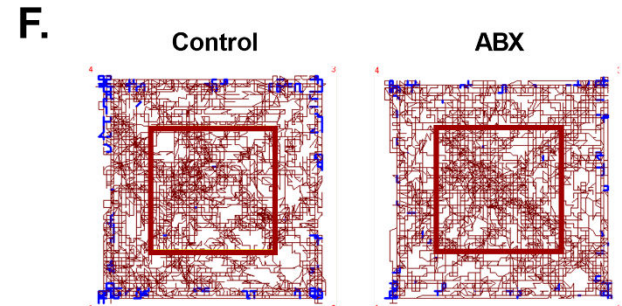
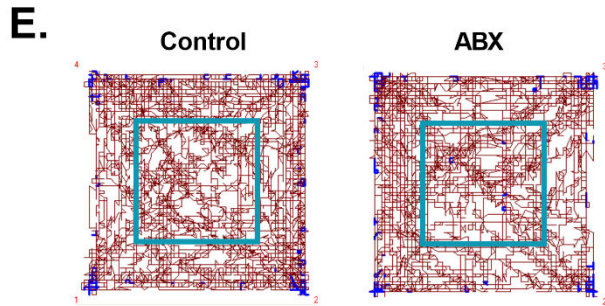
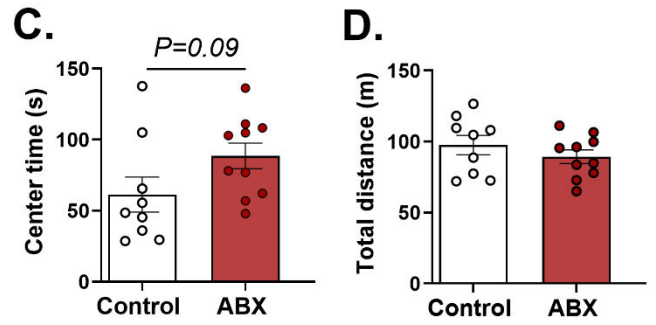
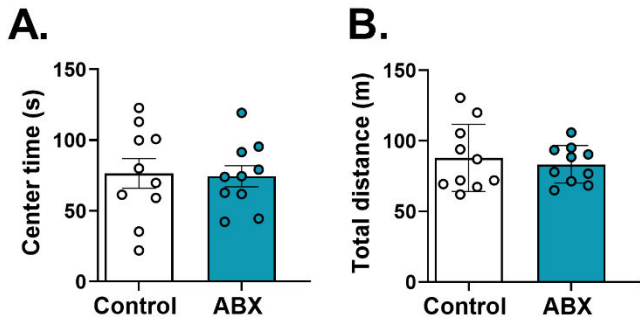


Figure S2. Perinatal ABX exposure does not lead to major changes in anxiety-like behavior in the high-light open-field test. (A-D) Time spent in the center zone of the testing box and distance travelled in the arena by control and perinatally ABX-exposed male (n = 9 per group) and female (n = 10 per group) juvenile offspring. (E, F) Representative tracks of movement patterns from control and perinatally ABX-exposed juvenile offspring. (G, H) Number of visits in each corner of the arena performed by control and perinatally ABX-exposed male (n = 9–10 per group) and female (n = 10 per group) juvenile offspring. All data are presented as means \pm SEM.

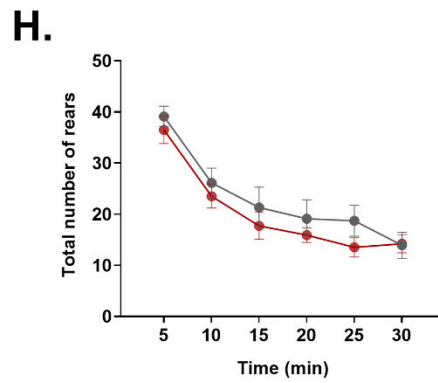
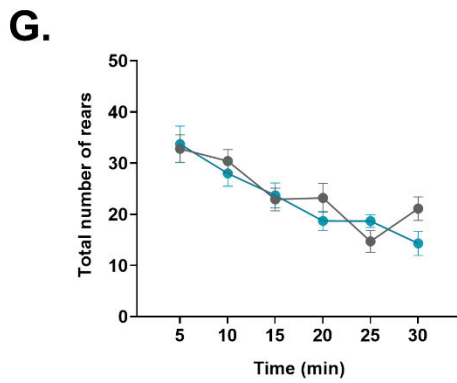
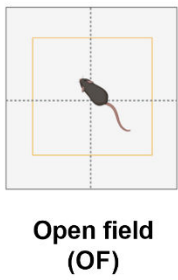
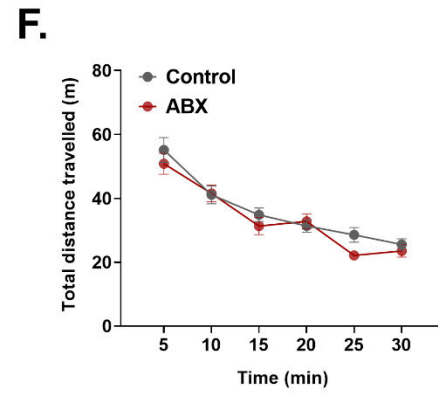
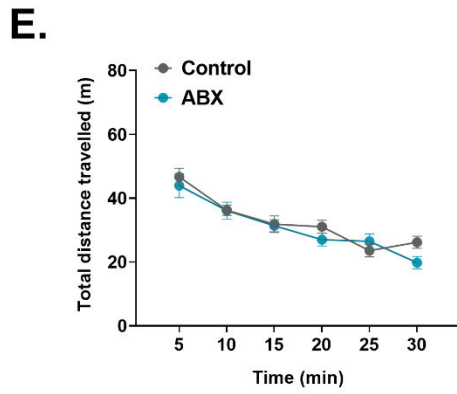
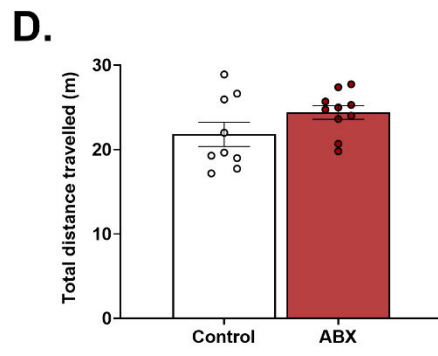
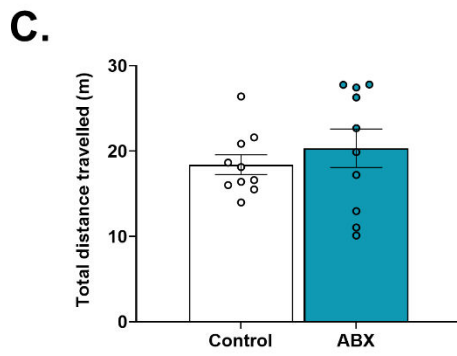
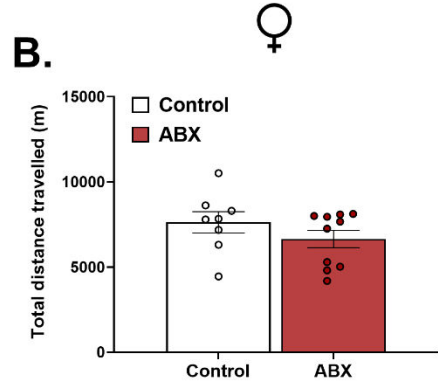
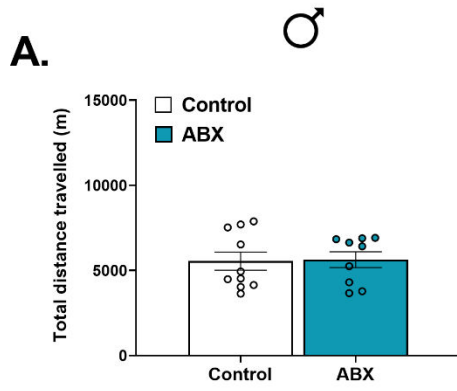
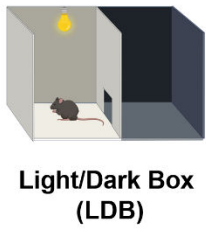
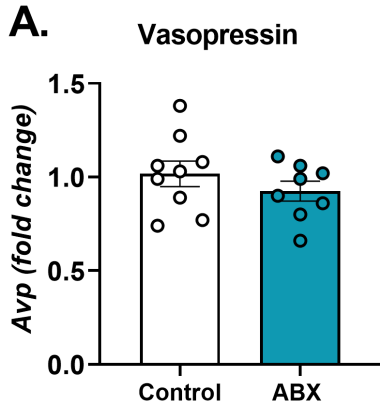


Figure S3. Perinatal ABX exposure does not impact the overall locomotor activity of juvenile offspring. (A-D) Total distance travelled during the light/dark box test (A, B) and the elevated plus maze test (C, D), by control and perinatally ABX-exposed male (n = 9–10 per group) and female (n = 9 per group) juvenile offspring. (E-H) Total distance over time (E, F) and total number of rears (e.g., exploratory behavior during which the mouse is in upward position on its hind legs) (G, H) during the open field test (under high-illumination) by control and perinatally ABX-exposed male (n = 10 per group) and female (n = 10 per group) juvenile offspring. All data are presented as means \pm SEM.



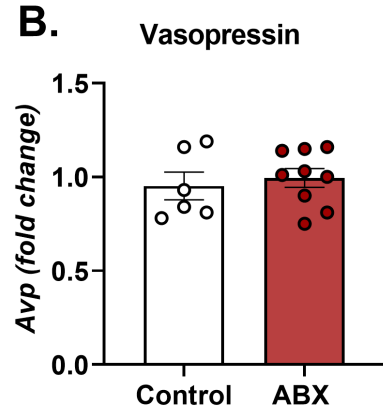
□ Control ■ ABX



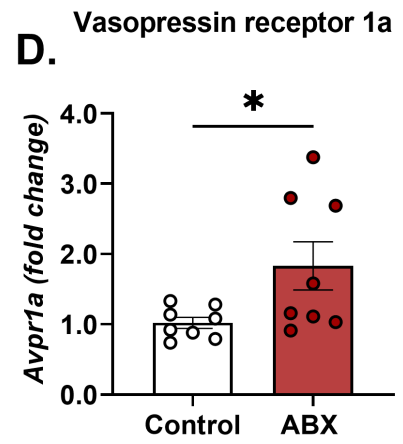
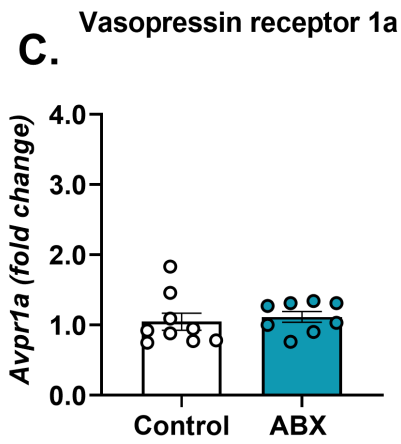
Hypothalamus



□ Control ■ ABX



Amygdala



Prefrontal cortex

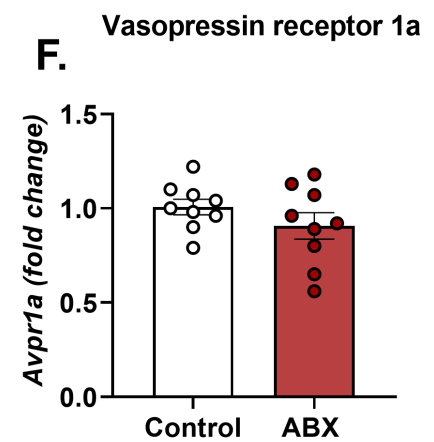
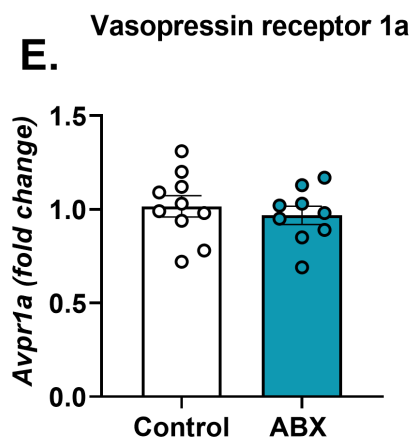


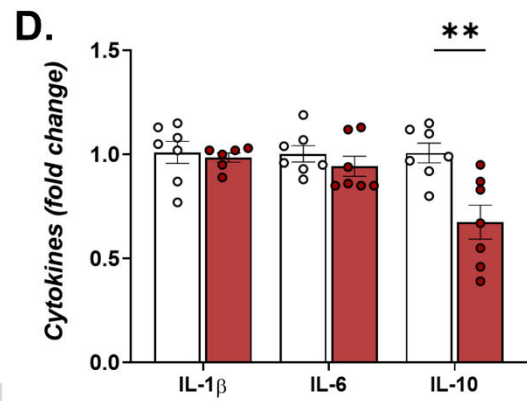
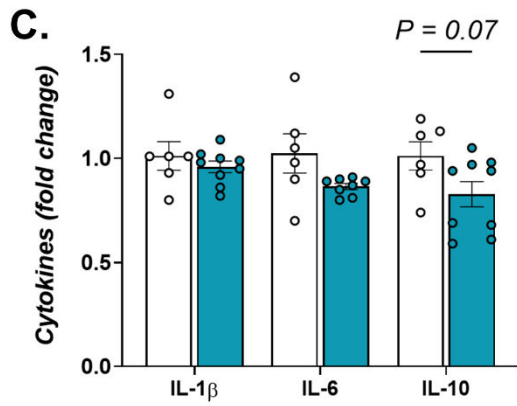
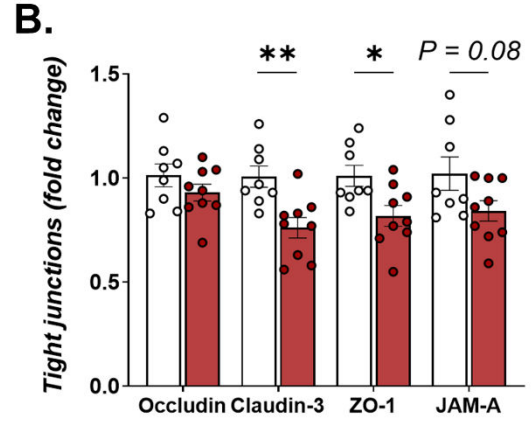
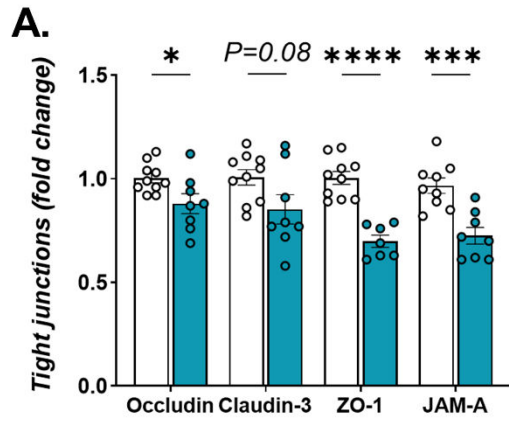
Figure S4. Perinatal ABX exposure alters Avpr1a gene expression in the amygdala of juvenile female offspring. (A, B) mRNA expression levels of arginine-vasopressin (Avp) in the hypothalamus of control and ABX-exposed male (n = 8–9 per group) and female (n = 6–9 per group) juvenile offspring. (C–F) mRNA expression levels of the arginine-vasopressin receptor 1a (Avpr1a) in the amygdala (C, D) and the prefrontal cortex (E, F) of control and perinatally ABX-exposed male (n = 8–10 per group) and female (n = 8–9 per group) juvenile offspring. All data are presented as means \pm SEM. *P < 0.05 compared to control female mice.



□ Control ■ ABX

□ Control ■ ABX

Prefrontal cortex



Colon

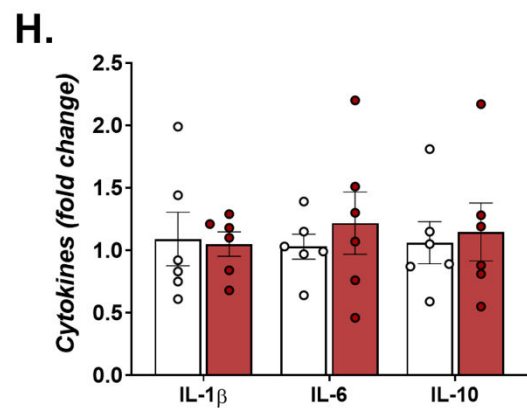
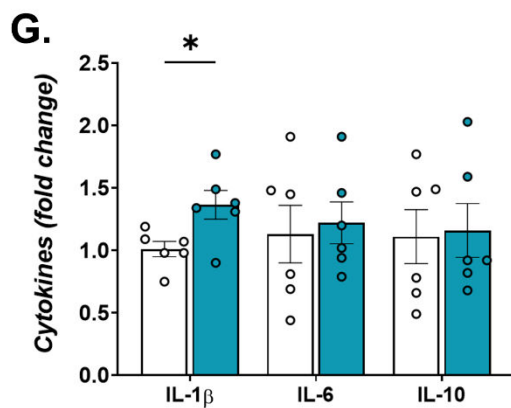
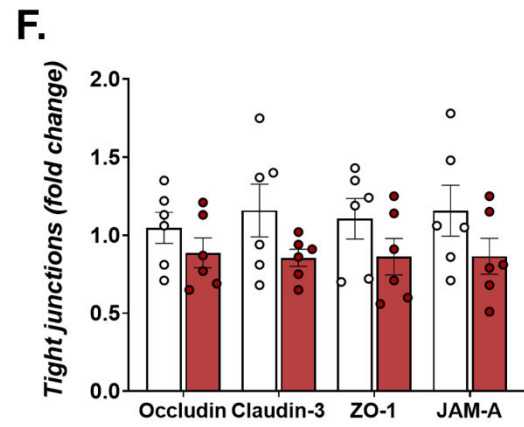
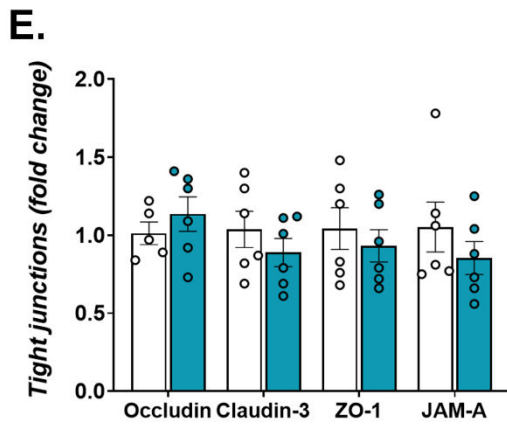


Figure S5. Tight junction proteins and cytokines mRNA levels are altered in the prefrontal cortex (PFC) and colon of offspring from ABX-treated dams, especially in males. (A-D) mRNA expression levels of tight junctions (e.g., occludin, claudin-3, zonula occludens 1 [ZO-1] and junctional adhesion molecule A [JAM-A]) (A, B) and cytokines (C, D) in the PFC of control and perinatally ABX-exposed male (n = 6–9 per group) and female (n = 7–9 per group) juvenile offspring. (E-H) mRNA levels of tight junctions (E-F) and cytokines (G, H) in the colon of control and perinatally ABX-exposed male (n = 6 per group) and female (n = 6 per group) juvenile offspring. All data are presented as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 compared to control offspring mice.

General Discussion & Perspectives

General Discussion & Perspectives

1. Potential link between key microbial species and neurobehavioral abnormalities

Our study revealed changes in the gut microbiota composition of offspring from dams exposed to a broad-spectrum antibiotic (ampicillin) during a narrow perinatal window. Particularly, we found that the relative abundance of the *Parabacteroides* genus is increased in perinatally antibiotic exposed offspring. In accordance with our findings, *Lynch and colleagues*²¹⁸ showed that a cocktail of antibiotics (including ampicillin) during narrow postnatal periods (either in the neonatal, pre-pubertal or post-pubertal periods) leads to an increase in this genus. By contrast, antibiotic-induced microbiota depletion during adolescence results in reduced *Parabacteroides* in male mice³⁷⁶. However, both of these reports demonstrated different behavioral outcomes following antibiotic exposure compared to our study, indicating that gut microbiota-induced behavioral changes in mice depend on a complex combination of factors, such as type of exposure (direct *vs.* indirect), the duration of treatment, the developmental window of exposure, the dose and type of antibiotic regimen. Taken together, these studies suggest that early life is a critical window of vulnerability for the establishment of the *Parabacteroides* genus. We further demonstrate that *Parabacteroides goldsteinii* is one of the most significantly altered bacterial species in the gut microbiota of perinatally-exposed offspring. Interestingly, patients with ASD display significant changes in the relative abundance of *Parabacteroides*^{385,386}, and decreased levels of *P. goldsteinii* have been previously reported in the male BTBR T+ *Itpr3^{fl}/J* (BTBR) ASD mouse model³⁸⁷. In fact, reduced levels of *P. goldsteinii* are positively correlated with sociability and social recognition, but negatively associated with anxiety- and compulsive-like behaviors in the BTBR mouse³⁸⁷, which is reminiscent of the behavioral profile of our antibiotic mouse model. By contrast to our study, some ASD mouse models showed an underrepresentation of *P. goldsteinii* in the maternal immune activation model (MIA)³⁸⁸. In particular, supplementation with the *P. goldsteinii* strain MTS01 dampens colonic inflammation in the MIA offspring, notably through the reduction of *Tlr4* expression, and restores their socio-emotional deficits³⁸⁸, suggesting that *P. goldsteinii* might contribute to the neurobehavioral development through the modulation of LPS-driven inflammatory pathways. Interestingly, we found a very moderate pro-inflammatory state in offspring from antibiotic-exposed dams, and one could hypothesize that the increased levels of

P. goldsteinii contributes to the control of inflammation in our antibiotic offspring mouse model. This effect might also be held by the regulation of neuropeptidergic systems relevant to socio-emotional behaviors. Indeed, it was found that the relative abundance of the *Parabacteroides* genus is positively correlated with plasma oxytocin levels³⁸⁷ and probiotic supplementation with *P. goldsteinii* upregulates *Avpr1a* and downregulates *Crh* expression in the hippocampus of MIA offspring³⁸⁸, possibly indicating that *P. goldsteinii* might potentiate pro-social neuropeptidergic systems functioning. In addition, another possible hypothesis would be that *Parabacteroides* species contribute to brain development and behavior via the GABAergic signaling pathway, as these bacteria are able to produce GABA (similarly to *Bacteroides* and *Escherichia* species) in the healthy human gut microbiota³⁸⁹. Overall, these observations indicate that early life establishment of *Parabacteroides* bacteria may be critical for neurodevelopment. Particularly, *P. goldsteinii* likely signals to the developing brain during the perinatal period, probably in brain regions expressing socially-relevant neuropeptidergic pathways, contributing to the development of socio-emotional behaviors relevant to ASD.

Similar to *Parabacteroides*, we report an increase in the relative abundance of *Akkermansia*, and particularly *A. muciniphila*, in the gut microbiota of perinatally-exposed juvenile offspring. This commensal bacterium is paramount for the intestinal mucus barrier by its ability to degrade mucins and contributes to its balance in the gut^{110,116}. Administration of *A. muciniphila* decreases chronic stress-induced depressive- and anxiety-like behaviors in mice^{390,391} through the modulation of the host metabolome, ultimately restoring neurotransmitter signaling pathways, HPA axis hyperactivity and the gut microbiota composition³⁹¹. As *A. muciniphila* relative abundance is increased in the gut microbiota of our perinatally-exposed offspring, this might explain, at least partly, the context-dependent decrease in anxiety-like behavior in male juvenile offspring following perinatal antibiotic exposure. Fascinatingly, *A. muciniphila* programs hippocampus-dependent cognition in early life³⁹², and this effect might be mediated by its outer membrane protein, Amuc-1100³⁹¹. *A. muciniphila* might also communicate with host inflammatory pathways and neurotransmitter signaling through its extracellular vesicles, that were reported to mitigate pro-inflammatory cytokines gene expression in both the colon and hippocampus of mice, and enhance the gut serotonin metabolism. Moreover, supplementation with the live *A. muciniphila* bacterium to a microbiota-depleted mouse model increases the relative abundance of phyla such as *Firmicutes* and *Bacilli*³⁹¹, pointing out that its presence might be required for the growth of some commensal bacteria, which could be another indirect pathway through which *A. muciniphila* influences neurobehavioral outcomes

in the mouse. Taken together, these observations indicate that, beyond its role in mucus barrier protection, *A. muciniphila* may use different signaling strategies to communicate with the host during early life and control emotional behaviors. Furthermore, as our findings indicate that the whole *Verruimicrobia* genus, that includes *A. muciniphila*, is more abundant in perinatally-exposed females compared to matched males, the male brain vulnerability to abnormal development could stem, at least in part, from differences in *A. muciniphila* abundance between females and males at the juvenile period. Interestingly, there is a lower abundance of *Akkermansia* bacteria in the gut microbiota of ASD children^{385,386}, probably confirming that changes in *Akkermansia* levels are involved in neuroatypical development. Of note, mouse depressive-like behaviors are characterized by a downregulated *Bdnf* expression, a decrease in circulating dopamine and serotonin, and an hyperactivation of the HPA axis³⁹¹, which are rescued by the supplementation with *A. muciniphila*^{390,391}. Altogether, these observations suggest that this bacterium may act as a promising psychobiotic, *i.e.*, a probiotic agent acting on the microbiota-gut-brain axis to ameliorate neurodevelopmental and neuropsychiatric disorders.

To date, the most studied bacterial genus for its beneficial effects over neurodevelopment and behaviors are *Lactobacilli*, and we report that *Lactobacillus murinus* is increased in the fecal microbiota of perinatally-exposed offspring, whereas no significant changes are found at the genus level. As mentioned in the general introduction, *Lactobacilli* are the most encountered bacterial species in the neonatal and pre-weaning gut microbiota, and are pivotal for the development of host health. Indeed, mono-colonization with *Lactobacillus murinus* strain HU-1 in offspring from antibiotic-depleted dams is sufficient to rescue neonatal motor defects and social deficits in juveniles, more markedly in males than in females³⁹³. The beneficial effects of *L. murinus* HU-1 on offspring postnatal behavior is mediated by its ability to interact with microglial life cycle and senescence in the juvenile PFC, indicating that *L. murinus* would be pivotal for the development of microglia in cortical layers that regulate the establishment of motor control and later, for the development of social behavior. However, as opposed to the report of *Lebovitz and colleagues*³⁹³, our antibiotic regimen used a single antibiotic during a narrow window of exposure, and leads to compositional changes within the gut microbiota rather than total microbiota depletion. Hence, complex interactions between other bacteria in our mouse model might mitigate the beneficial effects of *L. murinus* on social behavior in the juvenile period. This may also confirm that brain development and socio-emotional behaviors are shaped by various microbial cues, such as from *Akkermansia* or *Parabacteroides*, for

instance. Moreover, juvenile offspring from ampicillin-exposed dams in our report showed a very moderate inflammatory status as opposed to juveniles from antibiotic-depleted mothers displaying increased microglial reactivity and IL-1 β expression in the PFC³⁹³, thus suggesting that the presence of *L. murinus* in the gut microbiota of our juvenile offspring mice might confer a protection against neuroinflammation, but might not be sufficient to counteract other microbial cues over socio-emotional developmental outcomes and BBB permeability.

Importantly, our perinatal antibiotic regimen results in strong sex-specific differences in the gut microbiota composition, which are very similar to the gut microbiota phenotype of the BTBR ASD mouse model³⁹⁴. We note a decrease in microbial α -diversity only in perinatally-exposed females, with significant reductions in the *Blautia* genus, *Candidatus arthromitus* (a segmented filamentous bacterium) and *Bacteroides caecimuris*; whereas matched males, but not females, display increased relative abundance in *Bacteroides acidifaciens* and *Anaerotruncus colihominis*. We thus speculate that these sex-dependent compositional differences might play a role in the sex-biased neurobehavioral phenotype of perinatally-exposed offspring, and particularly the marked behavioral alterations in male offspring compared to females, which are reminiscent of ASD incidence in humans³⁹⁵. As such, similar to our perinatal antibiotic mouse model, BTBR mice display sex-specific alterations in their gut microbiota composition, especially in the *Bacteroides*, *Parabacteroides*, *Lactobacillus* and *Akkermansia* genera³⁹⁴. Similar to offspring from antibiotic-treated dams, the relative abundance of *Parabacteroides* is increased in both male and female BTBR animals; *Akkermansia* bacteria are less abundant in the BTBR females, whereas decreased levels of *Akkermansia* are visible in both sexes in our study. In addition, *Bacteroides* levels rise in both sexes in BTBR animals whereas we report an increase in *Bacteroides* only in perinatally-exposed males. In parallel, sex-specific gut microbiota alterations of BTBR animals are associated with an autistic-like phenotype that is more 'severe' than in our model. For instance, the gut inflammatory profile and the avoidance of social interactions are striking in BTBR mice, in both sexes; whereas we only show a moderate pro-inflammatory status and more subtle changes in social behavior in our perinatally-exposed juvenile offspring, especially in males. Finally, although many experimental factors are different between the aforementioned study of BTBR mice and ours (e.g., the age of testing, the genetic background and the use of different behavioral tests and measures), we both report changes in the same key bacterial endophenotypes and a male vulnerability to ASD-related neurobehavioral abnormalities. Likewise, the most frequently altered bacterial genera are *Parabacteroides*, *Akkermansia*, *Bacteroides* and *Lactobacillus* in ASD children^{385,386}, which is

in accordance with our findings and other results in NDD rodent models^{394,396}. However, the presence and/or relative abundance of these genera are variable between human studies and might depend on several confounding factors such as medication exposure or diet in ASD-diagnosed patients^{360,386}.

Altogether, these observations suggest that our perinatal antibiotic exposure in mice might serve as a relevant model for NDD, and particularly for ASD, since sex-specific alterations in key bacterial taxa of the gut microbiota are associated with significant impairments in male-prone brain molecular and early life socio-emotional behavior. Nevertheless, compared to other pre-clinical models of early life adversity^{179,182,187,213}, the autistic-like phenotype described in our study may be more moderate than other rodent models, and point out that studying the combination of several factors, as described in the study of *Rincel and colleagues*¹⁸², would add even more clinical relevance to our study.

2. Emerging microbiota-focused interventions to treat neurodevelopmental disorders

Beneficial effects of probiotics. The use of probiotics during the perinatal period ameliorates the infant gut microbiota fitness in both rodents^{215,216,397} and humans^{225,398}. Especially, *Lactobacilli* are the most abundant colonizers in the neonatal period¹¹⁶, and their use as probiotics is raising interest for the understanding, and eventually, the treatment of NDD. Although the exact mechanisms underlying the role of *Lactobacilli* or *Bifidobacteria* on brain development is still not well understood today, few studies started to report potential benefits for neurobehavioral development. In human infants, *Lactobacilli* probiotics were shown to be safe and have long-term health benefits in low-weight pre-term infants^{399,400}, which further encourages the use of prophylactic probiotics around birth in populations at risk for infections and antibiotic use in early life¹¹⁶. Firstly, *L. reuteri* would be a promising avenue for the treatment of ASD forms of sociability deficits in genetic, environmental or idiopathic ASD models^{81,187,401}. Indeed, the exposure to *L. reuteri* early in life rescues sociability and social recognition impairments that are common to all of these ASD models^{81,187,401}. Another study³⁵⁰ showed that *L. helveticus* strain CCFM1076 ameliorates ASD-relevant behaviors, such as social deficits, but also anxiety- and compulsive-like behaviors in the maternal valproic acid-exposed rat, another environmental ASD model. Perhaps even more relevant to our study are the findings of *Leclercq et al.*²¹⁶ and *Kayyal et al.*²¹⁵, respectively showing that early postnatal and perinatal exposure to *L. rhamnosus* JB-1 restores antibiotic-induced NDD-related

neurobehavioral alterations. As such, concurrent antibiotic and *L. rhamnosus* JB-1 probiotic supplementation correct the increased male anxiety-like behaviors and social deficits that are caused by perinatal antibiotic exposure ²¹⁶, which resembles to the behavioral profile of our perinatal antibiotic offspring model. Also, *L.rhamnosus* JB-1 supplementation following perinatal antibiotic exposure restores the gut microbiota composition on a long-term period, as well as rescuing cortical and hippocampal BBB integrity, neuroinflammation and corrects the levels of *Avpr1b* expression in the PFC ²¹⁶. By contrast, *L.rhamnosus* JB-1 supplementation after postnatal antibiotic exposure only correct social deficits of antibiotic-treated juveniles, concomitantly to the sex-dependent rescue of *Avpr1a* and *Avpr1b* expression in the PFC ²¹⁵. Thus, both the reports of *Leclercq et al.* ²¹⁶ and *Kayyal and coworkers* ²¹⁵ demonstrate that *L. rhamnosus* JB-1 rescue the sex-specific detrimental effects of antibiotic exposure on the microbiota composition and BBB integrity of offspring. Interestingly, neither antibiotic exposure or colonization with *L. rhamnosus* JB-1 change central *Oxtr* expression ²¹⁵, as opposed to our findings, which further confirms that the timing of exposure and the type of antibiotic influence the effects of the developing gut microbiota on the OXT system. Moreover, it seems that *L. rhamnosus* JB-1 postnatal effects on brain function and behavior is mediated by its interaction with Tregs and DCs, specifically in male offspring ²¹⁵, suggesting that *L. rhamnosus* JB-1 might modulate neuroimmune and neuroendocrine pathways to regulate socio-emotional behaviors. Nevertheless, *Leclercq and colleagues* ²¹⁶ did not investigate the effect of *L. rhamnosus* JB-1 on antibiotic-exposed offspring on the oxytocinergic system and neuroimmune pathways, and as they performed a longer antibiotic course than ours, we may wonder whether exposure to *L. rhamnosus* JB-1 during a narrow perinatal period would rescue abnormal OXTR system in the PFC, sex-specific alterations in BBB integrity and ASD-related behavioral impairments of offspring perinatally exposed to β -lactams (*e.g.*, penicillins, ampicillin). Although very informative about the potential signaling pathways by which gut commensals can interact with host gut-to-brain functioning, these studies highlight the fact that lactobacilli strains might not be a solution of all core behavioral symptoms of the disorder. Thus, one perspective of our study would be to test the postnatal supplementation of *L. rhamnosus* JB-1 on the microbiota-gut-brain axis outcomes in our mouse model. Also, the time of testing would be important to assess the longevity of potential benefits of *L. rhamnosus* JB-1 exposure, and thus we propose to test perinatally-exposed offspring in the short term (*e.g.*, at the juvenile period) and the long-term period (*e.g.*, in adulthood) for gut microbial seeding/microbiota establishment, as well as for the expression of the OXT/OXTR system and neurobehavioral

outcomes. Nonetheless, other key probiotic strains would also be of interest in our mouse model.

Indeed, *Bifidobacteria* probiotics participate to the establishment of neuronal circuits by improving synaptic formation, refinement and plasticity in the cortex, hippocampus and cerebellum in early postnatal life, ⁴⁰². The colonization of GF neonates until weaning with a *Bifidobacterium* consortium (*i.e.*, *B. longum* subsp. *infantis*, *B. breve*, *B. bifidum*, and *B. dentium*) replicate the positive effects of a whole FMT on synaptic-related gene expression and microglial reactivity ⁴⁰². As such, these *Bifidobacteria* species are sufficient for a proper gut microbiota colonization, the development of neuronal networks, and may influence microglial morphology and functions in early life. One may hypothesize that these *Bifidobacteria* species, *B. longum* subsp. *infantis*, *B. breve*, *B. bifidum*, and *B. dentium*, are key bacteria for the proper development of the infant microbiota-gut-brain axis and would represent a promising probiotic intervention in neonates at risk for NDD (*e.g.*, pre-terms and/or antibiotic-exposed infants), which etiology is hypothesized to originate from synaptic formation/pruning deficiencies in early life ⁴⁰³. Also, exposure to *Bifidobacterium pseudocatenulatum* strain CECT 7765 in the whole pre-weaning period limits the long-lasting excessive stress levels and anxiety-like behavior induced by postnatal maternal separation. These findings are in agreement with the report of *Sudo and colleagues* ⁶² demonstrating that neonatal *B. infantis* exposure attenuates the stress-induced hyperactivation of the HPA axis in mice. Similarly, *B. pseudocatenulatum* CECT 7765 decreases catecholaminergic neurotransmitter concentrations in the hypothalamus and gut of juvenile offspring, and restores serum and intestinal pro-inflammatory cytokine levels at P21. These neurobehavioral effects are probably mediated by the ability of *B. pseudocatenulatum* CECT 7765 to rescue gut microbiota alterations induced by maternal separation stress in juvenile and adult offspring, and by improving the glucocorticoid sensitivity in mesenteric lymph nodes ³⁹⁷, suggesting a potential effect of *B. pseudocatenulatum* CECT 7765 on neuroimmune and neuroendocrine pathways to dampen stress-related behaviors.

Moreover, it was recently shown that *Blautia stercoris* strain MRx0006 improves ASD-related behavioral alterations in the BTBR mouse model, including peer-to-peer social interactions and socio-sexual interest, as well as reducing compulsive- and anxiety-like behaviors ⁴⁰⁴. Strikingly, this specific bacterial strain modulates the expression of OXT and AVP neuropeptides, but not their receptors, in the hypothalamus. This live biotherapeutic strain also modifies the cecal metabolome of BTBR mice, notably circulating tryptophan and 5-HT levels ⁴⁰⁴. Altogether, this suggests that *Blautia stercoris* strain MRx0006 might alleviate the atypical

neurodevelopment and behavioral alterations of the BTBR mouse through the modulation of OXT and AVP and the 5-HT metabolism. As we demonstrate a sex-dependent decrease in the *Blautia* genus in perinatally-exposed offspring, we may wonder if *Blautia stercoris* strain MRx0006 exposure in our antibiotic mouse model would lead to a similar rescue in NDD-relevant behaviors.

Taken together, these studies indicate that selective probiotic strains may be promising non-invasive therapies for ASD-associated socio-emotional deficits. An interesting perspective to our antibiotic mouse model would be to alternate or combine the exposure to *Lactobacilli*, *Bifidobacteria* and *Blautia* in parallel with the maternal ampicillin treatment, during relevant time windows of the mouse gut postnatal colonization, and assess the same neurobehavioral outcomes in the neonatal (e.g., USV communication) and juvenile periods (social, anxiety-like and locomotor behaviors), together with OXT/OXTR gene expression and BBB integrity.

Minocycline, a ‘hopeful’ molecule for treating neuropsychiatric disorders and NDD.

Interestingly some broad-spectrum antibiotics, such as minocycline, are studied for their potential therapeutical effects on neuropsychiatric disorders⁴⁰⁵. In particular, minocycline is a broad-spectrum antibiotic of the tetracyclines family, that targets both Gram-negative and Gram-positive bacteria by the inhibition of prokaryotic ribosomes during the protein synthesis⁴⁰⁶. Besides its antimicrobial properties, minocycline has anti-inflammatory, anti-oxidant and neuroprotective effects. Due to its high lipophilicity, it can cross the BBB and might thus have a gut microbiota-independent effect on brain function^{405,406}. Besides its known antidepressant properties, increasing evidence demonstrates the potential therapeutic effects of minocycline on autism or fragile X syndrome, a genetic form of intellectual disability, which repetitive behaviors and social deficits are common to other neurodevelopmental conditions. In rodents, minocycline from weaning rescues social deficits, partially restores BBB permeability and the reduction of GI motility in prenatal valproic acid-exposed rat model of autism, while correcting the 5-HT imbalance in the gut and brain⁴⁰⁷. Due to its anti-inflammatory and anti-oxidative properties, minocycline inhibits microglial activation, and brain oxidation, thus limiting neuroinflammation^{405,407,408}, but whether these effects are mediated by the gut microbiota requires further investigation. In the autism/fragile X syndrome *Fmr1* KO model, post-weaning minocycline rescues the reduction of adult ultrasonic mating-oriented communication, by the attenuation of the metalloproteinase-9, a protein that cleaves extracellular matrix elements that is elevated in Fragile X syndrome patients⁴⁰⁸. Interestingly, minocycline rescues the social, locomotor and compulsive-like behaviors in BTBR mice by improving neuroinflammation and

hippocampal neurogenesis deficits⁴⁰⁹. Recently, one team explored the effects of minocycline on the microbiota-gut-brain axis functions in adult rats prone to anxiety-like behaviors⁴¹⁰. Their study revealed that a 22-day course with minocycline leads to drastic changes in the gut microbiota composition, with a more marked increase in butyrate-producing bacteria (*e.g.*, *Lachnospiraceae* and *Clostridiales* family XIII) and 3-OH-butyrate plasma levels in anxiety-prone rats compared to controls with normal anxiety levels. These microbial alterations are accompanied with sex-dependent antidepressant effects, as expected, and reduced microglial activation in the infralimbic and prelimbic PFC in high anxiety-prone rats⁴¹⁰. This important study highlights the potentiality of minocycline to modulate the gut microbiota and its metabolites to mitigate neuroinflammation in the PFC and neurobehavioral outcomes in general. As such, this antibiotic might represent a novel therapeutic strategy for NDD, including autism or the fragile X syndrome. Nonetheless, as minocycline is highly lipophilic and can cross easily the BBB⁴⁰⁶, its effects might also be independent of the gut microbiota. Thus, future studies focusing on this molecule should make a clear distinction between microbiota-dependent and microbiota-independent effects. In humans, a pilot study in ASD children using minocycline suggests that the antibiotic ameliorates serum pro-inflammatory chemokine (*e.g.*, CXCL8) levels and circulating neurotrophic factors (*e.g.*, BDNF) in the serum and cerebrospinal fluid, as well as a tendency for decreasing serum metalloproteinase-7 levels. Despite the lack of behavioral changes in these children after minocycline exposure, and due to the low number of participants, we may still wonder about the clinical relevance of minocycline on NDD or neurological disorders.

Altogether, these observations indicate that additional studies are needed to understand the efficacy, safety and mechanism of action of minocycline in NDD patients, and investigate notably the microbial and neurobehavioral outcomes of early life exposure to this molecule. Although minocycline, and tetracyclines in general, do not have the same structure and mechanism of action as β -lactams (*e.g.*, ampicillin and penicillin), the use of this molecule alone in NDD models or patients raises the interesting question of whether antibiotic-induced perturbations of the gut microbiota are always detrimental for brain development and the establishment of socio-emotional behaviors.

3. Biological pathways implicated in the crosstalk between the gut microbiota and the brain: Potential implications for early life antibiotic exposure

A vagal communication between gut microbes and the brain: implications for NDD.

Pioneer studies^{80,81} started to delineate a potential vagal gut-to-brain mechanism underlying the behavioral benefits from the gut microbiota. For instance, subdiaphragmatic vagotomy in mice prevents the activation of the mesenteric nerve bundle by *L. rhamnosus* JB-1⁸⁰, and suppresses the beneficial effects of *L. reuteri* on social behavior in the *Shank3B*-deficient ASD mouse⁸¹. Thus, these findings suggest that some commensal bacteria communicate with the gut vagal afferents that regulate socially-relevant brain regions in mice. Moreover, it was shown that *L. reuteri* potentiates social-driven behaviors through its ability to activate the hypothalamic oxytocinergic system projecting to reward dopaminergic neurons in the mesolimbic VTA¹⁸⁷, in diverse ASD genetic^{81,401}, environmental and idiopathic^{81,187} mouse models, altogether suggesting a vagal and neuroendocrine-dependent pathway by which *Lactobacilli* would modulate the development of socially-relevant brain regions. Recently, a complete vagal gut-to-striatum neuronal pathway regulating motivational and reward behaviors has been described in the mouse⁷⁸. In particular, the right vagal sensory ganglion, a ganglionic relay receiving right stomachal and duodenal vagal afferents (also called nodose ganglia) projects to the dorsal vagal complex (including the nucleus of solitary tract; NTS), itself triggering dopamine release through the parabrachial-nigrostriatal dopaminergic pathway, resulting in increased motivation for food in mice. Fascinatingly, this study also presents an NTS-mediated projection of gut vagal afferents to dopaminergic and non-dopaminergic cells of the PVN, the main hypothalamic nucleus of oxytocinergic, vasopressinergic and *Crh*-expressing neurons. In light of these findings, we may hypothesize a direct vagal pathway from the gut to the PVN to modulate the oxytocinergic-dopaminergic reward system involved in mouse social behaviors. However, the exact mechanism(s) by which such *Lactobacilli* strains signal to the vagal gut-to-brain axis are not well understood. Additional mechanistic investigations established that *L. reuteri* does not activate the vagal oxytocinergic pathways through the restoration of the gut microbiota composition⁸¹, or the adaptive immune system (*i.e.*, B and T lymphocytes) in the gut⁴¹¹. Instead, it has been proposed that *L. reuteri* signals to the vagus nerve via bacterial metabolites in the intestinal compartment, such as tetrahydrobiopterin (BH4), that derives from a co-factor involved in the synthesis of neurotransmitters in the gut⁴¹¹. In that direction, it was evidenced that the selective *Lactobacilli*

strain *L. helveticus* CCFM1076 can interact with the 5-HT neurotransmitter pathway, to modulate the oxytocinergic signaling networks, and ameliorates ASD-relevant behaviors in the prenatal valproic acid-exposed rat offspring³⁵⁰. Overall, these findings likely indicate a vagal gut-to-brain pathway by which gut microbes modulate the neuropeptidergic systems, including the oxytocinergic signaling, for the development and function of socio-emotional behaviors. Furthermore, it is not excluded that gut commensals act on the vagal gut-to-brain pathway through their microbial metabolites, produced within the gut compartment.

Microbial metabolites in the development of the microbiota-gut-brain-behavior axis: implications for NDD. Increasing evidence from clinical and pre-clinical studies shows that neuroatypical development is also linked to changes in the levels of microbial metabolites^{72,412}. Indeed, plasma levels of indolelactate, and other tryptophan metabolites, in ASD children, are decreased compared to neurotypical children^{72,412}. Importantly, these microbial-derived metabolites can cross the BBB^{240,413} and are detected as early as the neonatal period (*i.e.*, from P3) in the mouse forebrain, and are either the products of both host and microbiome metabolisms or purely originating from the commensal microbiota metabolism⁴¹³. Across the postnatal development, microbial-derived metabolites undergo differential dynamic temporal patterns in the mouse forebrain. For example, imidazole propionate levels peak at P21⁴¹³ and decrease thereafter in adulthood. As imidazole propionate, and other microbial maternal bacterial metabolites, such as hippurate, 5-aminovalerate and 3-indoxyl sulfate, accelerate thalamo-cortical axonogenesis in the fetal mouse brain mice²²⁸, this supports the idea that developmental patterns of microbial-derived metabolites have an implication for proper brain development. Another pivotal study has showed indeed that microbial metabolization of 4-EP into 4-EPS is related to altered oligodendrocyte maturation, myelination defects and behavioral alterations²⁴⁰. More precisely, colonization with 4-EPS-producing bacteria results in differential brain activation and functional connectivity between specific CNS regions such as the paraventricular thalamus, the BNST, the amygdala, the medial PFC and the hypothalamus (although to a lesser extent)²⁴⁰. In parallel, 4-EP-supplemented mice display an ASD-relevant behavioral phenotype, including increased anxiety-like behavior, and tendencies for self-repetitive behavior and social deficits (*i.e.*, decreased social interactions and USV communication), without showing any changes in the locomotor activity²⁴⁰. This behavioral profile is reminiscent of our perinatal antibiotic offspring model, suggesting a possible effect of microbial-derived 4-EPS, and other microbial-derived metabolites, on the onset of socio-emotional impairments in mice. Of note, another tyrosine microbial metabolite, *p*-cresol, also

affects the myelination process in the PFC, resulting in decreased social behavior and increase depressive-like behavior ²⁴¹. However, both *p*-cresol and 4-EPS are produced by *Clostridiales* commensal species, such as *Lachnospiraceae* and *Ruminococcaceae*, which are in higher levels in ASD patients ^{72,412}. By comparison, we demonstrate that *Lachnospiraceae* bacterium relative abundance is decreased in our perinatal antibiotic-exposed offspring, which suggests that increased anxiety-like behavior observed in our male offspring from antibiotic-exposed dams is unlikely to be related with changes in levels of neuroactive 4-EPS or *p*-cresol.

In addition, higher plasma levels of steroid hormones, especially androgens, are reported in ASD children ⁷². One might think that increased levels of circulating androgens may be linked to the increased vulnerability of males for NDD ⁴¹⁴, as demonstrated by large clinical evidence and supported by numerous pre-clinical studies, such as ours. Moreover, a very recent clinical report ⁴¹² showed that fecal metabolomic profiles of children at high risk for ASD are different from low-risk children, especially with decreased levels of the inhibitory neurotransmitter GABA at 5 months and 36 months of age, which are positively and negatively correlated with *Bifidobacteria* and *Clostridia* relative abundance, respectively. *In vitro* analyses in this study ⁴¹² showed that *Bifidobacteria* species produce GABA while *Clostridia* bacteria consume it, suggesting that the imbalanced levels of *Bifidobacteria* and *Clostridia* in high ASD risk children contribute to the differences in fecal GABA levels in children with elevated ASD likelihood. Interestingly, elevated risk of ASD in these children is associated with learning deficits at 36 months of age, but not before ⁴¹². In addition, forebrain levels of some neurotransmitters, and their microbial-derived metabolites, are changing with age. GABA forebrain levels increase with age, reaching their maximum levels at P21 ²²⁸, corresponding to the GABA switch neuronal maturation event. Altogether, this suggests that differences in *Bifidobacteria* and *Clostridia* levels in the gut microbiota in early life might underlie the differences in neurotransmitter signaling (*e.g.*, GABA or 5-HT) along the microbiota-gut-brain axis, which would have an implication for the onset and/or progression of NDD, such as ASD.

4. The peptidoglycan signaling pathway in the development of neuropeptidergic systems: implications for neurodevelopmental disorders

In our study, we demonstrate a significant decrease in *Oxtr*, but not *Avpr1a* or *Crhr1*, expression in the PFC of juvenile offspring from perinatal antibiotic-treated dams. However, the potential causal relationship between gut microbiota compositional changes with *Oxtr* gene expression

alterations after our perinatal antibiotic treatment should be investigated in future studies. Recently, it was shown that post-weaning antibiotic depletion in mice leads to decreased *Oxtr* gene expression in the frontal cortex, similarly to offspring from perinatal antibiotic-exposed offspring in our report, and is associated with a decreased gene expression of MyD88 in the frontal cortex of antibiotic-exposed mice ³⁴⁸. Of note, MyD88 is a downstream molecule of several innate immune molecular pathways, that are triggered by bacterial surface molecules, such as lipopolysaccharides and peptidoglycans ⁴¹⁵. In particular, peptidoglycans (PGN) are highly-conserved molecular fragments, or ‘motifs’, that are present on the bacterial cell wall of both Gram-positive and Gram-negative bacteria ^{416,417}. Chemically, these bacterial-derived molecules consist of complex polymers of polysaccharides and amino acid chains ^{42,44,45}. As bacteria divide and grow, they can release PGN motifs across the whole organism ⁴¹⁵. Thus, one might speculate for a possible innate immune-dependent pathway by which gut microbes in early development communicate with brain neuropeptidergic systems, including the oxytocinergic system, and particularly the MyD88 molecular signaling recognizing bacterial surface molecules such as PGN.

Relationship between β -lactams antibiotics and peptidoglycans. Aminopenicillins, including ampicillin, target both Gram-positive and Gram-negative bacteria, by inhibiting an enzyme involved in the last step of PGN synthesis ⁴¹⁸. However, a recent report demonstrates that the use of β -lactam antibiotics, particularly ampicillin, induce a significant increase in PGN fragments release in the gut lumen of exposed mice, termed as a ‘PGN storm’ by the authors, from six hours after the antibiotic treatment ⁴¹⁹. Although the dose of ampicillin used in this study is eight times higher than the dose we used in our experiments, it might suggest a potential increase in the quantity of released PGN fragments in the gut microbiota of our exposed dams. As maternal-derived PGN motifs can cross the placental barrier and affect the cortical neuroproliferation in the fetal brain, later influencing postnatal behavioral outcomes ²²⁷, we can hypothesize that maternal-derived PGN fragments released after our perinatal ampicillin treatment impacts the offspring gut microbiota during the prenatal period. In addition, it was shown that ampicillin exposure in adult female mice results in increased levels of PGN levels in their feces, more markedly after ampicillin treatment than any other β -lactam antibiotic ⁴¹⁹. As such, we may speculate that PGN compounds in the feces from ampicillin-treated mothers might be another source of PGN exposure in neonatal offspring, although the ‘external’ stability of such compounds requires further investigation.

The central PGN signaling and socio-emotional behaviors. Mammalian hosts recognize disseminating PGN motifs through pattern recognition receptors (PRR) of the innate immune system ^{415,416,420}. In particular, PRR that specifically recognize PGN include the NOD-like receptors (NOD1 and NOD2), the peptidoglycan-recognition proteins (PGLYRP1-4) and peptidoglycan transporters (PepT1 for instance), together referred to as peptidoglycan-sensing molecules (or PGN-sensing molecules) ^{74,420}. As already mentioned, gut microbiota-derived PGN disseminate from the gut lumen into the bloodstream, and regulates the host immune system in the intestinal ⁵⁷ and extraintestinal compartments, such as the bone marrow ⁴²¹. For instance, *Clarke and colleagues* ⁴²¹ showed that some PGN motifs activate NOD1, which primes the killing function of bone marrow-derived neutrophils towards pathogens ⁴²¹. Similarly, the NOD1 signaling pathway is required in early life for the induction of ILFs in the mouse ileum and colon, via the recognition of PGNs motifs from Gram-negative bacteria ⁵⁷. On the other hand, NOD2 is necessary for both the control of gut microbiota composition and the protection from pathogen invasion at the intestinal epithelial barrier ^{422,423}. These observations points out to a microbiota-gut-immune crosstalk mediated by bacterial-derived PGN fragments, which contributes to the development of the immune and intestinal systems. More recently, *Arentsen and colleagues* ²²⁰ showed for the first time that PGN motifs can translocate directly to the developing mouse brain, and may serve as signaling molecules between the gut microbiota and the healthy CNS. Also, *Humann and colleagues* ²²⁷ showed a placental transfer of PGN fragments from the maternal gut microbiota to the fetal brain, that has an impact on cortical development and postnatal behavior, altogether suggesting an effect of bacterial PGN on the perinatal brain development. In the postnatal developing brain, PGN levels increase in an age-dependent manner, and are recognized by PGN-sensing molecules, such as *Pglyrp1-4*, *Nod1-2* and the PGN transporter *PepT1*, that are expressed in various neuronal populations ²²⁰. More importantly, *Pglyrp2* gene deletion in mice leads to social deficits, reminiscent of some ASD-relevant rodent models ²²⁰, including our perinatal antibiotic mouse model. Altogether, these studies propose a PGN signaling pathway as an emerging mechanism underlying the contribution of the gut microbiota on the development of brain and behaviors relevant to NDD.

In addition, others ³⁵⁶ demonstrated a potential role of *Nod1* and *Nod2* in the regulation of stress behaviors through the modulation of tryptophan and 5-HT signaling pathway and the activation of the HPA stress system. Indeed, constitutive deletion of both *Nod1* and *Nod2* leads to disturbed intestinal tryptophan and 5-HT signaling in the mouse, impacting central tryptophan and 5-HT

availability in the brain. In addition, the absence of NOD-like receptors leads to GI physiological impairments, HPA axis overactivation after stress, as well as cognitive and emotional deficits³⁵⁶. The authors found specifically that intestinal *Nod1* influences, at least partially, the hippocampal control of the HPA axis stress response and overall mood and cognition, whereas *Nod2* rather regulates the GI function. Altogether, these important results suggest that the PGN-NOD-like receptors signaling, originating from the intestinal epithelium, regulates stress and emotional responses, by acting both on the intestinal and the central 5-HT system³⁵⁶. However, the role of NOD-like receptors on stress-dependent regulation of mood and cognition might not only be attributable to their intestinal expression but also to central expression.

Very recently, *Gabanyi and colleagues*⁴²⁴ has identified a specific population of GABAergic neurons expressing *Nod2* in the arcuate (ARC) and dorsomedial (DMH) nuclei of the hypothalamus. They showed that PGN motifs specifically recognized by NOD2, called muropeptides (MDP), can derive from the gut microbiota and translocate from the gut to the brain through the bloodstream, mirroring previous findings^{220,425}. Furthermore, *Nod2* conditional KO in GABAergic neurons resulted in higher food consumption and weight gain, as well as dysregulated thermoregulation and nesting behaviors in females, but not males, which is exacerbated with age. By contrast, MDP exposure activates NOD2-expressing GABAergic interneurons, ultimately decreasing neuronal firing activity in the ARC and DMH, both controlling body thermoregulation and satiety in female mice. These findings reveal that MDP recruits NOD2 in inhibitory GABAergic neurons of the hypothalamus to control satiety and body metabolism in a sex- and age-dependent manner. Interestingly, the total absence of *Nod2* in the adult mouse triggers anxiety-like behavior in both sexes, although in a more marked manner in females compared to male counterparts⁴²⁴, further supporting the existence of a central PGN signaling pathway regulating emotional behaviors. Noteworthy, *Nod2* is expressed in the PVN, SON, SCH of the hypothalamus⁴²⁴ and *Nod2* is co-expressed with the hypothalamic orexigenic neuropeptide Y within the ARC⁴²⁴. Thus, we may hypothesize that *Nod2* is co-expressed with *Oxt*, *Avp*, *Crh*, or all three neuropeptides, within the hypothalamus (e.g., in the PVN, SON or SCH), which would underlie the influence of NOD2 on anxiety-like behavior. Although it is already established that *Nod2* is expressed in the hypothalamus of adult and old mice⁴²⁴, the ontogenesis of both *Nod1* and *Nod2* in the whole developing hypothalamus has not been investigated yet. If *Nod1* hypothalamic expression is demonstrated, one may assess its potential co-expression with socio-emotional-relevant OXT and AVP neuropeptides. During

early life development, the gene expression of PGN-sensing molecules describes region- and sex-specific dynamic temporal patterns in the striatum, PFC, hippocampus and cerebellum, during the mouse postnatal development²²⁰. For example, *Nod1* shows a peak of expression at P21 (*i.e.*, the time of weaning in mice) in both the striatum and cerebellum (although only in males), which coincides with precise time windows of synaptogenesis and synaptic pruning^{129,222,223}. By contrast, *Nod2* ontogenesis is variable between the striatum, cerebellum and prefrontal cortex of the same mice during early life²²⁰. The dynamic temporal patterns of *Nod1* and *Nod2* expression may have a functional implication for brain development and behavior, since another PGN-sensing molecule, PGLYRP2, was shown to differentially regulate cognitive and socio-emotional behaviors in an age-dependent manner. Indeed, constitutive *Pglyrp2* deletion in the mouse leads to alterations in social behavior at the juvenile period²²⁰, while aged *Pglyrp2*-deficient mice rather show anxiety-like behavior and locomotor impairments⁴²⁵. To test this hypothesis, we may take advantage of *Nod1* and/or *Nod2*-deficient animals for the assessment of the expression levels of *Oxt*, *Avp* and their respective receptors within the developing brain, as well as measuring associated social, anxiety-like and cognitive behavioral outcomes. Another plausible model to answer this question would be the early life supplementation with PGN motifs that are specifically recognized by NOD1 ligands (*i.e.*, diaminopimelic acid compounds) and NOD2 ligands (*i.e.*, muropeptides, MDP), and assess the potential effects over socio-emotional and cognitive behavioral outcomes. To establish a more precise link between commensal PGN and neuropeptidergic system development, one may quantify the expression of *Oxt*, *Avp* and their receptors, *Oxtr*, *Avpr1a* and *Avpr1b*, in brain regions relevant for the regulation of social and affective behaviors *i.e.*, the hypothalamus, the PFC or the amygdala. These complementary animal models would help to decipher the potential implications of a central PGN-Nod1/2 signaling pathway(s) by which gut colonizing microbes contribute to the development of the mouse brain and associated behaviors, and probably more towards a better understanding of the influence of bacterial components over typical and atypical neurodevelopment.

Interestingly, *Nod1* and *Nod2* gene expression are also found in non-neuronal populations such as microglia^{424,426}, oligodendrocytes⁷⁴, and central endothelial cells⁴²⁴, thus suggesting that the central PGN signaling pathway might involve several brain cell networks for their influence on brain function and behavior.

5. General Conclusion

In summary, we show that ampicillin-induced alterations of the maternal gut microbiota during a narrow critical window around birth is sufficient to alter the gut microbiota and socio-emotional behaviors in the offspring. As increasingly reported, these antibiotic-induced physiological and neurobehavioral alterations are strongly sex-dependent, as it affects more specifically males over females. Importantly, we demonstrate that exposure to a low-dose of ampicillin during a narrow perinatal window is sufficient to disturb the development of social- and emotional-driven behaviors from the neonatal life until the juvenile period, particularly in males. However, perinatal antibiotic-induced neurobehavioral alterations are associated with altered oxytocinergic signaling and BBB integrity in the PFC in both sexes, which suggests a potential resilience of the female brain towards external stimuli during development. Furthermore, these effects may be mediated by an imbalance in the relative abundance of several gut bacterial species, such as *Parabacteroides goldsteinii*, *Akkermansia muciniphila* and *Lactobacillus murinus*, which are shown to influence neurobehavioral outcomes in several neurodevelopmental disorders-relevant mouse models. Nevertheless, the exact mechanisms, by which these commensal bacteria communicate with the gut-brain axis for the establishment of socio-emotional behaviors are not fully elucidated yet. Based upon preliminary evidence, we propose a common pathway in which bacterial PGN, derived from the gut microbiota, modulate the shaping of the oxytocinergic signaling pathway and the BBB formation during early life, in social- and emotional-relevant brain regions, such as the PFC. We further postulate that, during the early life period, microbiota-derived PGN bind to their sensing molecules, such as NOD1 or NOD2, within the brain, which downstream signaling activation interacts with developing neuropeptidergic systems, including the oxytocinergic and vasopressinergic systems.

Bibliography

Bibliography

1. Robinson, J. M. *Invisible friends: how microbes shape our lives and the world around us*. (Pelagic Publishing, 2023).
2. Leidy, J. *A flora and fauna within living animals*. (Smithsonian Institution, 1853). doi:10.5962/bhl.title.56319.
3. Gilbert, J. A. & Neufeld, J. D. Life in a World without Microbes. *PLoS Biol.* **12**, e1002020 (2014).
4. Roberts, C. S. William Beaumont, the Man and the Opportunity. in *Clinical Methods: The History, Physical, and Laboratory Examinations* (eds. Walker, H. K., Hall, W. D. & Hurst, J. W.) (Butterworths, 1990).
5. Diaz Heijtz, R. & Swann, J. Editorial overview: CNS diseases and the microbiome. *Curr. Opin. Pharmacol.* **48**, x–xii (2019).
6. Eckburg, P. B. *et al.* Diversity of the Human Intestinal Microbial Flora. *Science* **308**, 1635–1638 (2005).
7. MetaHIT Consortium *et al.* A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**, 59–65 (2010).
8. The Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012).
9. Sender, R., Fuchs, S. & Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLOS Biol.* **14**, e1002533 (2016).
10. MetaHIT Consortium *et al.* An integrated catalog of reference genes in the human gut microbiome. *Nat. Biotechnol.* **32**, 834–841 (2014).
11. Schroeder, B. O. & Bäckhed, F. Signals from the gut microbiota to distant organs in physiology and disease. *Nat. Med.* **22**, 1079–1089 (2016).
12. Rinninella, E. *et al.* What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* **7**, 14 (2019).
13. Oliveira, R. A. & Pamer, E. G. Assembling symbiotic bacterial species into live therapeutic consortia that reconstitute microbiome functions. *Cell Host Microbe* **31**, 472–484 (2023).

14. Donaldson, G. P., Lee, S. M. & Mazmanian, S. K. Gut biogeography of the bacterial microbiota. *Nat. Rev. Microbiol.* **14**, 20–32 (2016).
15. Sekirov, I., Russell, S. L., Antunes, L. C. M. & Finlay, B. B. Gut Microbiota in Health and Disease. *Physiol. Rev.* **90**, 859–904 (2010).
16. Almeida, A. *et al.* A new genomic blueprint of the human gut microbiota. *Nature* **568**, 499–504 (2019).
17. Oren, A. & Garrity, G. M. Valid publication of the names of forty-two phyla of prokaryotes. *Int. J. Syst. Evol. Microbiol.* **71**, (2021).
18. Sommer, F., Anderson, J. M., Bharti, R., Raes, J. & Rosenstiel, P. The resilience of the intestinal microbiota influences health and disease. *Nat. Rev. Microbiol.* **15**, 630–638 (2017).
19. Contijoch, E. J. *et al.* Gut microbiota density influences host physiology and is shaped by host and microbial factors. *eLife* **8**, e40553 (2019).
20. Gustafsson, B. E., Daft, F. S., McDaniel, E. G., Smith, J. C. & Fitzgerald, R. J. Effects of Vitamin K-Active Compounds and Intestinal Microorganisms in Vitamin K-Deficient Germfree Rats. *J. Nutr.* **78**, 461–468 (1962).
21. Magnúsdóttir, S., Ravcheev, D., De Crécy-Lagard, V. & Thiele, I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. *Front. Genet.* **6**, (2015).
22. Oliphant, K. & Allen-Vercoe, E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome* **7**, 91 (2019).
23. Yano, J. M. *et al.* Indigenous Bacteria from the Gut Microbiota Regulate Host Serotonin Biosynthesis. *Cell* **161**, 264–276 (2015).
24. Hubbard, B. P. *et al.* Continuous directed evolution of DNA-binding proteins to improve TALEN specificity. *Nat. Methods* **12**, 939–942 (2015).
25. Clarke, G. *et al.* The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol. Psychiatry* **18**, 666–673 (2013).
26. Reigstad, C. S. *et al.* Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. *FASEB J.* **29**, 1395–1403 (2015).

27. Rowland, I. *et al.* Gut microbiota functions: metabolism of nutrients and other food components. *Eur. J. Nutr.* **57**, 1–24 (2018).
28. Begley, M., Gahan, C. G. M. & Hill, C. The interaction between bacteria and bile. *FEMS Microbiol. Rev.* **29**, 625–651 (2005).
29. Bergman, E. N. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* **70**, 567–590 (1990).
30. De Vadder, F. *et al.* Microbiota-Generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits. *Cell* **156**, 84–96 (2014).
31. Bäckhed, F. *et al.* The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci.* **101**, 15718–15723 (2004).
32. Sonnenburg, J. L. *et al.* Glycan Foraging in Vivo by an Intestine-Adapted Bacterial Symbiont. *Science* **307**, 1955–1959 (2005).
33. Desai, M. S. *et al.* A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. *Cell* **167**, 1339–1353.e21 (2016).
34. Zhang, L. S. & Davies, S. S. Microbial metabolism of dietary components to bioactive metabolites: opportunities for new therapeutic interventions. *Genome Med.* **8**, 46 (2016).
35. Maslowski, K. M. *et al.* Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* **461**, 1282–1286 (2009).
36. De Filippo, C. *et al.* Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci.* **107**, 14691–14696 (2010).
37. Ley, R. E. *et al.* Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci.* **102**, 11070–11075 (2005).
38. Gill, S. R. *et al.* Metagenomic Analysis of the Human Distal Gut Microbiome. *Science* **312**, 1355–1359 (2006).
39. Valdes, A. M., Walter, J., Segal, E. & Spector, T. D. Role of the gut microbiota in nutrition and health. *BMJ* k2179 (2018) doi:10.1136/bmj.k2179.
40. Muniz, L. R., Knosp, C. & Yeretssian, G. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Front. Immunol.* **3**, (2012).

41. Zheng, D., Liwinski, T. & Elinav, E. Interaction between microbiota and immunity in health and disease. *Cell Res.* **30**, 492–506 (2020).
42. Schaedler, R. W., Dubos, R. & Costello, R. THE DEVELOPMENT OF THE BACTERIAL FLORA IN THE GASTROINTESTINAL TRACT OF MICE. *J. Exp. Med.* **122**, 59–66 (1965).
43. Uribe, A., Alam, M., Johansson, O., Midtvedt, T. & Theodorsson, E. Microflora modulates endocrine cells in the gastrointestinal mucosa of the rat. *Gastroenterology* **107**, 1259–1269 (1994).
44. Hooper, L. V. *et al.* Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. *Science* **291**, 881–884 (2001).
45. Lutgendorff, F., Akkermans, L. & Soderholm, J. The Role of Microbiota and Probiotics in Stress-Induced Gastrointestinal Damage. *Curr. Mol. Med.* **8**, 282–298 (2008).
46. Dieterich, W., Schink, M. & Zopf, Y. Microbiota in the Gastrointestinal Tract. *Med. Sci.* **6**, 116 (2018).
47. Suzuki, T. Regulation of the intestinal barrier by nutrients: The role of tight junctions. *Anim. Sci. J.* **91**, e13357 (2020).
48. Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S. & Medzhitov, R. Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. *Cell* **118**, 229–241 (2004).
49. Sommer, F. & Bäckhed, F. The gut microbiota — masters of host development and physiology. *Nat. Rev. Microbiol.* **11**, 227–238 (2013).
50. McVey Neufeld, K. A., Mao, Y. K., Bienenstock, J., Foster, J. A. & Kunze, W. A. The microbiome is essential for normal gut intrinsic primary afferent neuron excitability in the mouse: Microbiome essential for enteric nervous system. *Neurogastroenterol. Motil.* **25**, 183-e88 (2013).
51. De Vadder, F. *et al.* Gut microbiota regulates maturation of the adult enteric nervous system via enteric serotonin networks. *Proc. Natl. Acad. Sci.* **115**, 6458–6463 (2018).
52. Geuking, M. B., Köller, Y., Rupp, S. & McCoy, K. D. The interplay between the gut microbiota and the immune system. *Gut Microbes* **5**, 411–418 (2014).
53. Kawamoto, S. *et al.* The Inhibitory Receptor PD-1 Regulates IgA Selection and Bacterial Composition in the Gut. *Science* **336**, 485–489 (2012).

54. Suzuki, K. *et al.* Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc. Natl. Acad. Sci.* **101**, 1981–1986 (2004).
55. Franchi, L., Muñoz-Planillo, R. & Núñez, G. Sensing and reacting to microbes through the inflammasomes. *Nat. Immunol.* **13**, 325–332 (2012).
56. Abo-Shaban, T. *et al.* Issues for patchy tissues: defining roles for gut-associated lymphoid tissue in neurodevelopment and disease. *J. Neural Transm.* **130**, 269–280 (2023).
57. Bouskra, D. *et al.* Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature* **456**, 507–510 (2008).
58. Tanaka, M. & Nakayama, J. Development of the gut microbiota in infancy and its impact on health in later life. *Allergol. Int.* **66**, 515–522 (2017).
59. Engevik, M. A. *et al.* Immunomodulation of dendritic cells by *Lactobacillus reuteri* surface components and metabolites. *Physiol. Rep.* **9**, (2021).
60. Ivanov, I. I. *et al.* Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. *Cell* **139**, 485–498 (2009).
61. Luczynski, P. *et al.* Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. *Int. J. Neuropsychopharmacol.* **19**, pyw020 (2016).
62. Sudo, N. *et al.* Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice: Commensal microbiota and stress response. *J. Physiol.* **558**, 263–275 (2004).
63. Heijtz, R. D. *et al.* Normal gut microbiota modulates brain development and behavior. *Proc. Natl. Acad. Sci.* **108**, 3047–3052 (2011).
64. Neufeld, K. M., Kang, N., Bienenstock, J. & Foster, J. A. Reduced anxiety-like behavior and central neurochemical change in germ-free mice: Behavior in germ-free mice. *Neurogastroenterol. Motil.* **23**, 255-e119 (2011).
65. Bercik, P. *et al.* The Intestinal Microbiota Affect Central Levels of Brain-Derived Neurotrophic Factor and Behavior in Mice. *Gastroenterology* **141**, 599-609.e3 (2011).
66. Hsiao, E. Y. *et al.* Microbiota Modulate Behavioral and Physiological Abnormalities Associated with Neurodevelopmental Disorders. *Cell* **155**, 1451–1463 (2013).

67. Desbonnet, L. *et al.* Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain. Behav. Immun.* **48**, 165–173 (2015).
68. Needham, B. D. *et al.* A gut-derived metabolite alters brain activity and anxiety behaviour in mice. *Nature* **602**, 647–653 (2022).
69. Carlson, A. L. *et al.* Infant Gut Microbiome Associated With Cognitive Development. *Biol. Psychiatry* **83**, 148–159 (2018).
70. Kang, D.-W. *et al.* Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. *Microbiome* **5**, 10 (2017).
71. Kang, D.-W. *et al.* Long-term benefit of Microbiota Transfer Therapy on autism symptoms and gut microbiota. *Sci. Rep.* **9**, 5821 (2019).
72. Needham, B. D. *et al.* Plasma and Fecal Metabolite Profiles in Autism Spectrum Disorder. *Biol. Psychiatry* **89**, 451–462 (2021).
73. Stewart Campbell, A. *et al.* Safety and target engagement of an oral small-molecule sequestrant in adolescents with autism spectrum disorder: an open-label phase 1b/2a trial. *Nat. Med.* **28**, 528–534 (2022).
74. Gonzalez-Santana, A. & Diaz Heijtz, R. Bacterial Peptidoglycans from Microbiota in Neurodevelopment and Behavior. *Trends Mol. Med.* **26**, 729–743 (2020).
75. Kaelberer, M. M. *et al.* A gut-brain neural circuit for nutrient sensory transduction. *Science* **361**, eaat5236 (2018).
76. Fülling, C., Dinan, T. G. & Cryan, J. F. Gut Microbe to Brain Signaling: What Happens in Vagus.... *Neuron* **101**, 998–1002 (2019).
77. Cryan, J. F. *et al.* The Microbiota-Gut-Brain Axis. *Physiol. Rev.* **99**, 1877–2013 (2019).
78. Han, W. *et al.* A Neural Circuit for Gut-Induced Reward. *Cell* **175**, 665–678.e23 (2018).
79. Bravo, J. A. *et al.* Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci.* **108**, 16050–16055 (2011).

80. Perez-Burgos, A. *et al.* Psychoactive bacteria *Lactobacillus rhamnosus* (JB-1) elicits rapid frequency facilitation in vagal afferents. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **304**, G211–G220 (2013).
81. Sgritta, M. *et al.* Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. *Neuron* **101**, 246–259.e6 (2019).
82. Tennaoune, N. *et al.* Bacterial ClpB heat-shock protein, an antigen-mimetic of the anorexigenic peptide α -MSH, at the origin of eating disorders. *Transl. Psychiatry* **4**, e458–e458 (2014).
83. Breton, J. *et al.* Gut Commensal *E. coli* Proteins Activate Host Satiety Pathways following Nutrient-Induced Bacterial Growth. *Cell Metab.* **23**, 324–334 (2016).
84. Heiss, C. N. *et al.* The gut microbiota regulates hypothalamic inflammation and leptin sensitivity in Western diet-fed mice via a GLP-1R-dependent mechanism. *Cell Rep.* **35**, 109163 (2021).
85. Vagnerová, K. *et al.* Interactions Between Gut Microbiota and Acute Restraint Stress in Peripheral Structures of the Hypothalamic–Pituitary–Adrenal Axis and the Intestine of Male Mice. *Front. Immunol.* **10**, 2655 (2019).
86. Huo, R. *et al.* Microbiota Modulate Anxiety-Like Behavior and Endocrine Abnormalities in Hypothalamic-Pituitary-Adrenal Axis. *Front. Cell. Infect. Microbiol.* **7**, 489 (2017).
87. Vodička, M. *et al.* Microbiota affects the expression of genes involved in HPA axis regulation and local metabolism of glucocorticoids in chronic psychosocial stress. *Brain. Behav. Immun.* **73**, 615–624 (2018).
88. Crumeyrolle-Arias, M. *et al.* Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. *Psychoneuroendocrinology* **42**, 207–217 (2014).
89. Liu, Y., Sanderson, D., Mian, M. F., McVey Neufeld, K.-A. & Forsythe, P. Loss of vagal integrity disrupts immune components of the microbiota-gut-brain axis and inhibits the effect of *Lactobacillus rhamnosus* on behavior and the corticosterone stress response. *Neuropharmacology* **195**, 108682 (2021).
90. Wu, W.-L. *et al.* Microbiota regulate social behaviour via stress response neurons in the brain. *Nature* **595**, 409–414 (2021).

91. Fung, T. C., Olson, C. A. & Hsiao, E. Y. Interactions between the microbiota, immune and nervous systems in health and disease. *Nat. Neurosci.* **20**, 145–155 (2017).
92. Lee, L.-F. *et al.* IL-7 Promotes T_H1 Development and Serum IL-7 Predicts Clinical Response to Interferon- β in Multiple Sclerosis. *Sci. Transl. Med.* **3**, (2011).
93. Bharwani, A. *et al.* Structural & functional consequences of chronic psychosocial stress on the microbiome & host. *Psychoneuroendocrinology* **63**, 217–227 (2016).
94. D’Mello, C. *et al.* Probiotics Improve Inflammation-Associated Sickness Behavior by Altering Communication between the Peripheral Immune System and the Brain. *J. Neurosci.* **35**, 10821–10830 (2015).
95. Erny, D. *et al.* Host microbiota constantly control maturation and function of microglia in the CNS. *Nat. Neurosci.* **18**, 965–977 (2015).
96. Möhle, L. *et al.* Ly6Chi Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis. *Cell Rep.* **15**, 1945–1956 (2016).
97. O’Riordan, K. J. *et al.* Short chain fatty acids: Microbial metabolites for gut-brain axis signalling. *Mol. Cell. Endocrinol.* **546**, 111572 (2022).
98. Braniste, V. *et al.* The gut microbiota influences blood-brain barrier permeability in mice. *Sci. Transl. Med.* **6**, (2014).
99. Frost, G. *et al.* The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nat. Commun.* **5**, 3611 (2014).
100. Tolhurst, G. *et al.* Short-Chain Fatty Acids Stimulate Glucagon-Like Peptide-1 Secretion via the G-Protein–Coupled Receptor FFAR2. *Diabetes* **61**, 364–371 (2012).
101. Byrne, C. S. *et al.* Increased colonic propionate reduces anticipatory reward responses in the human striatum to high-energy foods. *Am. J. Clin. Nutr.* **104**, 5–14 (2016).
102. Hoyles, L. *et al.* Microbiome–host systems interactions: protective effects of propionate upon the blood–brain barrier. *Microbiome* **6**, 55 (2018).
103. Yamawaki, Y. *et al.* Sodium butyrate abolishes lipopolysaccharide-induced depression-like behaviors and hippocampal microglial activation in mice. *Brain Res.* **1680**, 13–38 (2018).

104. Patnala, R., Arumugam, T. V., Gupta, N. & Dheen, S. T. HDAC Inhibitor Sodium Butyrate-Mediated Epigenetic Regulation Enhances Neuroprotective Function of Microglia During Ischemic Stroke. *Mol. Neurobiol.* **54**, 6391–6411 (2017).
105. Wang, Z. *et al.* In Silico Prediction of Blood-Brain Barrier Permeability of Compounds by Machine Learning and Resampling Methods. *ChemMedChem* **13**, 2189–2201 (2018).
106. Gheorghe, C. E. *et al.* Focus on the essentials: tryptophan metabolism and the microbiome-gut-brain axis. *Curr. Opin. Pharmacol.* **48**, 137–145 (2019).
107. Jaglin, M. *et al.* Indole, a Signaling Molecule Produced by the Gut Microbiota, Negatively Impacts Emotional Behaviors in Rats. *Front. Neurosci.* **12**, 216 (2018).
108. Wei, G. Z. *et al.* Tryptophan-metabolizing gut microbes regulate adult neurogenesis via the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci.* **118**, e2021091118 (2021).
109. Osadchiy, V. *et al.* Correlation of tryptophan metabolites with connectivity of extended central reward network in healthy subjects. *PLOS ONE* **13**, e0201772 (2018).
110. Wang, S. *et al.* Maternal Vertical Transmission Affecting Early-life Microbiota Development. *Trends Microbiol.* **28**, 28–45 (2020).
111. Mady, E. A. *et al.* Impact of the mother’s gut microbiota on infant microbiome and brain development. *Neurosci. Biobehav. Rev.* **150**, 105195 (2023).
112. Perez-Muñoz, M. E., Arrieta, M.-C., Ramer-Tait, A. E. & Walter, J. A critical assessment of the “sterile womb” and “in utero colonization” hypotheses: implications for research on the pioneer infant microbiome. *Microbiome* **5**, 48 (2017).
113. Rackaityte, E. *et al.* Viable bacterial colonization is highly limited in the human intestine in utero. *Nat. Med.* **26**, 599–607 (2020).
114. Mishra, A. *et al.* Microbial exposure during early human development primes fetal immune cells. *Cell* **184**, 3394-3409.e20 (2021).
115. Moore, R. E. & Townsend, S. D. Temporal development of the infant gut microbiome. *Open Biol.* **9**, 190128 (2019).
116. Xiao, L. & Zhao, F. Microbial transmission, colonisation and succession: from pregnancy to infancy. *Gut* **72**, 772–786 (2023).

117. Stanislowski, M. A. *et al.* Pre-pregnancy weight, gestational weight gain, and the gut microbiota of mothers and their infants. *Microbiome* **5**, 113 (2017).
118. Koren, O. *et al.* Host Remodeling of the Gut Microbiome and Metabolic Changes during Pregnancy. *Cell* **150**, 470–480 (2012).
119. Romero, R. *et al.* The vaginal microbiota of pregnant women who subsequently have spontaneous preterm labor and delivery and those with a normal delivery at term. *Microbiome* **2**, 18 (2014).
120. Zhang, X. *et al.* Variation of the Vaginal Microbiome During and After Pregnancy in Chinese Women. *Genomics Proteomics Bioinformatics* **20**, 322–333 (2022).
121. Rodríguez, J. M. *et al.* The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb. Ecol. Health Dis.* **26**, (2015).
122. Jašarević, E., Howard, C. D., Masic, A. M., Beiting, D. P. & Bale, T. L. Stress during pregnancy alters temporal and spatial dynamics of the maternal and offspring microbiome in a sex-specific manner. *Sci. Rep.* **7**, 44182 (2017).
123. Korpela, K. *et al.* Intestinal microbiota development and gestational age in preterm neonates. *Sci. Rep.* **8**, 2453 (2018).
124. Yassour, M. *et al.* Strain-Level Analysis of Mother-to-Child Bacterial Transmission during the First Few Months of Life. *Cell Host Microbe* **24**, 146-154.e4 (2018).
125. Stewart, C. J. *et al.* Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature* **562**, 583–588 (2018).
126. De Andrés, J. *et al.* Physiological Translocation of Lactic Acid Bacteria during Pregnancy Contributes to the Composition of the Milk Microbiota in Mice. *Nutrients* **10**, 14 (2017).
127. Bäckhed, F. *et al.* Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host Microbe* **17**, 690–703 (2015).
128. Schloss, P. D. *et al.* Stabilization of the murine gut microbiome following weaning. *Gut Microbes* **3**, 383–393 (2012).
129. Kalbermatter, C., Fernandez Trigo, N., Christensen, S. & Ganai-Vonarburg, S. C. Maternal Microbiota, Early Life Colonization and Breast Milk Drive Immune Development in the Newborn. *Front. Immunol.* **12**, 683022 (2021).

130. Al Nabhani, Z. & Eberl, G. Imprinting of the immune system by the microbiota early in life. *Mucosal Immunol.* **13**, 183–189 (2020).
131. Brodin, P. Immune-microbe interactions early in life: A determinant of health and disease long term. *Science* **376**, 945–950 (2022).
132. Gomez De Agüero, M. *et al.* The maternal microbiota drives early postnatal innate immune development. *Science* **351**, 1296–1302 (2016).
133. Lim, A. I. *et al.* Prenatal maternal infection promotes tissue-specific immunity and inflammation in offspring. *Science* **373**, eabf3002 (2021).
134. Round, J. L. & Mazmanian, S. K. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* **9**, 313–323 (2009).
135. Jung, J., Surh, C. D. & Lee, Y. J. Microbial Colonization at Early Life Promotes the Development of Diet-Induced CD8 $\alpha\beta$ Intraepithelial T Cells. *Mol. Cells* **42**, 313–320 (2019).
136. Bain, C. C. *et al.* Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat. Immunol.* **15**, 929–937 (2014).
137. Sefik, E. *et al.* Individual intestinal symbionts induce a distinct population of ROR γ ⁺ regulatory T cells. *Science* **349**, 993–997 (2015).
138. Al Nabhani, Z. *et al.* A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult. *Immunity* **50**, 1276-1288.e5 (2019).
139. Olszak, T. *et al.* Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. *Science* **336**, 489–493 (2012).
140. Wesemann, D. R. *et al.* Microbial colonization influences early B-lineage development in the gut lamina propria. *Nature* **501**, 112–115 (2013).
141. Constantinides, M. G. *et al.* MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science* **366**, eaax6624 (2019).
142. Henrick, B. M. *et al.* Bifidobacteria-mediated immune system imprinting early in life. *Cell* **184**, 3884-3898.e11 (2021).
143. Feehley, T. *et al.* Healthy infants harbor intestinal bacteria that protect against food allergy. *Nat. Med.* **25**, 448–453 (2019).

144. Knoop, K. A. *et al.* Microbial antigen encounter during a preweaning interval is critical for tolerance to gut bacteria. *Sci. Immunol.* **2**, eaao1314 (2017).
145. Patel, R. M. *et al.* Probiotic Bacteria Induce Maturation of Intestinal Claudin 3 Expression and Barrier Function. *Am. J. Pathol.* **180**, 626–635 (2012).
146. Kiu, R. *et al.* Bifidobacterium breve UCC2003 Induces a Distinct Global Transcriptomic Program in Neonatal Murine Intestinal Epithelial Cells. *iScience* **23**, 101336 (2020).
147. Abo, H. *et al.* Erythroid differentiation regulator-1 induced by microbiota in early life drives intestinal stem cell proliferation and regeneration. *Nat. Commun.* **11**, 513 (2020).
148. Brun, P. *et al.* Toll-Like Receptor 2 Regulates Intestinal Inflammation by Controlling Integrity of the Enteric Nervous System. *Gastroenterology* **145**, 1323–1333 (2013).
149. Caputi, V. *et al.* Antibiotic-induced dysbiosis of the microbiota impairs gut neuromuscular function in juvenile mice: Gut dysbiosis impairs neuromuscular function. *Br. J. Pharmacol.* **174**, 3623–3639 (2017).
150. Yu, D.-H. *et al.* Postnatal epigenetic regulation of intestinal stem cells requires DNA methylation and is guided by the microbiome. *Genome Biol.* **16**, 211 (2015).
151. Beaumont, M. *et al.* Gut microbiota derived metabolites contribute to intestinal barrier maturation at the suckling-to-weaning transition. *Gut Microbes* **11**, 1268–1286 (2020).
152. Ganz, J. & Ratcliffe, E. M. Who’s talking to whom: microbiome-enteric nervous system interactions in early life. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **324**, G196–G206 (2023).
153. Collins, J., Borojevic, R., Verdu, E. F., Huizinga, J. D. & Ratcliffe, E. M. Intestinal microbiota influence the early postnatal development of the enteric nervous system. *Neurogastroenterol. Motil.* **26**, 98–107 (2014).
154. Kabouridis, P. S. *et al.* Microbiota Controls the Homeostasis of Glial Cells in the Gut Lamina Propria. *Neuron* **85**, 289–295 (2015).
155. Diaz Heijtz, R. Fetal, neonatal, and infant microbiome: Perturbations and subsequent effects on brain development and behavior. *Semin. Fetal. Neonatal Med.* **21**, 410–417 (2016).
156. Borre, Y. E. *et al.* Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol. Med.* **20**, 509–518 (2014).

157. Hansen, C. H. F. *et al.* Mode of Delivery Shapes Gut Colonization Pattern and Modulates Regulatory Immunity in Mice. *J. Immunol.* **193**, 1213–1222 (2014).
158. Shao, Y. *et al.* Stunted microbiota and opportunistic pathogen colonization in caesarean-section birth. *Nature* **574**, 117–121 (2019).
159. Vandenplas, Y. *et al.* Factors affecting early-life intestinal microbiota development. *Nutrition* **78**, 110812 (2020).
160. Dominguez-Bello, M. G., Blaser, M. J., Ley, R. E. & Knight, R. Development of the Human Gastrointestinal Microbiota and Insights From High-Throughput Sequencing. *Gastroenterology* **140**, 1713–1719 (2011).
161. Stokholm, J. *et al.* Delivery mode and gut microbial changes correlate with an increased risk of childhood asthma. *Sci. Transl. Med.* **12**, eaax9929 (2020).
162. Zachariassen, L. F. *et al.* Cesarean Section Induces Microbiota-Regulated Immune Disturbances in C57BL/6 Mice. *J. Immunol.* **202**, 142–150 (2019).
163. Korpela, K. *et al.* Maternal Fecal Microbiota Transplantation in Cesarean-Born Infants Rapidly Restores Normal Gut Microbial Development: A Proof-of-Concept Study. *Cell* **183**, 324-334.e5 (2020).
164. Juárez, I., Gratton, A. & Flores, G. Ontogeny of altered dendritic morphology in the rat prefrontal cortex, hippocampus, and nucleus accumbens following Cesarean delivery and birth anoxia. *J. Comp. Neurol.* **507**, 1734–1747 (2008).
165. Castillo-Ruiz, A., Mosley, M., Jacobs, A. J., Hoffiz, Y. C. & Forger, N. G. Birth delivery mode alters perinatal cell death in the mouse brain. *Proc. Natl. Acad. Sci.* **115**, 11826–11831 (2018).
166. Morais, L. H. *et al.* Enduring Behavioral Effects Induced by Birth by Caesarean Section in the Mouse. *Curr. Biol.* **30**, 3761-3774.e6 (2020).
167. Castillo-Ruiz, A., Cisternas, C. D., Sturgeon, H. & Forger, N. G. Birth triggers an inflammatory response in the neonatal periphery and brain. *Brain. Behav. Immun.* **104**, 122–136 (2022).
168. Curran, E. A. *et al.* Birth by caesarean section and school performance in Swedish adolescents- a population-based study. *BMC Pregnancy Childbirth* **17**, 121 (2017).

169. O’Neill, S. M. *et al.* Birth by Caesarean Section and the Risk of Adult Psychosis: A Population-Based Cohort Study. *Schizophr. Bull.* **42**, 633–641 (2016).
170. Dinan, T. G. *et al.* Altered stress responses in adults born by Caesarean section. *Neurobiol. Stress* **16**, 100425 (2022).
171. Ma, J. *et al.* Comparison of gut microbiota in exclusively breast-fed and formula-fed babies: a study of 91 term infants. *Sci. Rep.* **10**, 15792 (2020).
172. Ratsika, A., Codagnone, M. C., O’Mahony, S., Stanton, C. & Cryan, J. F. Priming for Life: Early Life Nutrition and the Microbiota-Gut-Brain Axis. *Nutrients* **13**, 423 (2021).
173. Moszak, M., Szulińska, M. & Bogdański, P. You Are What You Eat—The Relationship between Diet, Microbiota, and Metabolic Disorders—A Review. *Nutrients* **12**, 1096 (2020).
174. Wood, H. *et al.* Breastfeeding promotes early neonatal regulatory T-cell expansion and immune tolerance of non-inherited maternal antigens. *Allergy* **76**, 2447–2460 (2021).
175. Fichter, M. *et al.* Breast milk contains relevant neurotrophic factors and cytokines for enteric nervous system development. *Mol. Nutr. Food Res.* **55**, 1592–1596 (2011).
176. O’Mahony, S. M. *et al.* The enduring effects of early-life stress on the microbiota–gut–brain axis are buffered by dietary supplementation with milk fat globule membrane and a prebiotic blend. *Eur. J. Neurosci.* **51**, 1042–1058 (2020).
177. Tarr, A. J. *et al.* The prebiotics 3’Sialyllactose and 6’Sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: Evidence for effects on the gut–brain axis. *Brain. Behav. Immun.* **50**, 166–177 (2015).
178. Quigley, M. A. *et al.* Breastfeeding is Associated with Improved Child Cognitive Development: A Population-Based Cohort Study. *J. Pediatr.* **160**, 25–32 (2012).
179. Golubeva, A. V. *et al.* Prenatal stress-induced alterations in major physiological systems correlate with gut microbiota composition in adulthood. *Psychoneuroendocrinology* **60**, 58–74 (2015).
180. Gur, T. L. *et al.* Prenatal stress affects placental cytokines and neurotrophins, commensal microbes, and anxiety-like behavior in adult female offspring. *Brain. Behav. Immun.* **64**, 50–58 (2017).
181. Gur, T. L. *et al.* Prenatal stress disrupts social behavior, cortical neurobiology and commensal microbes in adult male offspring. *Behav. Brain Res.* **359**, 886–894 (2019).

182. Rincel, M. *et al.* Multi-hit early life adversity affects gut microbiota, brain and behavior in a sex-dependent manner. *Brain. Behav. Immun.* **80**, 179–192 (2019).
183. Choi, G. B. *et al.* The maternal interleukin-17a pathway in mice promotes autism-like phenotypes in offspring. *Science* **351**, 933–939 (2016).
184. Pearson-Leary, J., Eacret, D. & Bhatnagar, S. Interleukin-1 α in the ventral hippocampus increases stress vulnerability and inflammation-related processes. *Stress* **23**, 308–317 (2020).
185. National Institute of Mental Health. Autism Spectrum Disorder (NIH Publication No. 22-MH-8084). (2023).
186. Rosin, S. *et al.* A preliminary study of gut microbiome variation and HPA axis reactivity in healthy infants. *Psychoneuroendocrinology* **124**, 105046 (2021).
187. Buffington, S. A. *et al.* Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* **165**, 1762–1775 (2016).
188. Bruce-Keller, A. J. *et al.* Maternal obese-type gut microbiota differentially impact cognition, anxiety and compulsive behavior in male and female offspring in mice. *PLOS ONE* **12**, e0175577 (2017).
189. Gohir, W. *et al.* Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother’s periconceptional diet. *Gut Microbes* **6**, 310–320 (2015).
190. Wang, J. *et al.* Dysbiosis of maternal and neonatal microbiota associated with gestational diabetes mellitus. *Gut* **67**, 1614–1625 (2018).
191. Galley, J. D., Bailey, M., Kamp Dush, C., Schoppe-Sullivan, S. & Christian, L. M. Maternal Obesity Is Associated with Alterations in the Gut Microbiome in Toddlers. *PLoS ONE* **9**, e113026 (2014).
192. Xie, R. *et al.* Maternal High Fat Diet Alters Gut Microbiota of Offspring and Exacerbates DSS-Induced Colitis in Adulthood. *Front. Immunol.* **9**, 2608 (2018).
193. Kimura, I. *et al.* Maternal gut microbiota in pregnancy influences offspring metabolic phenotype in mice. *Science* **367**, eaaw8429 (2020).
194. Haute Autorité de Santé, H. A. S. *Label de la HAS - Prise en charge du nouveau-né à risque d’infection néonatale bactérienne précoce (≥ 34 SA).* <https://www.has->

- sante.fr/jcms/c_2803349/fr/label-de-la-has-prise-en-charge-du-nouveau-ne-a-risque-d-infection-neonatale-bacterienne-precoce-34-sa (2017).
195. Nogacka, A. M. *et al.* Early microbiota, antibiotics and health. *Cell. Mol. Life Sci.* **75**, 83–91 (2018).
 196. Nyangahu, D. D. *et al.* Disruption of maternal gut microbiota during gestation alters offspring microbiota and immunity. *Microbiome* **6**, 124 (2018).
 197. Gonzalez-Perez, G. & Lamousé-Smith, E. S. N. Gastrointestinal Microbiome Dysbiosis in Infant Mice Alters Peripheral CD8+ T Cell Receptor Signaling. *Front. Immunol.* **8**, (2017).
 198. Cox, L. M. *et al.* Altering the Intestinal Microbiota during a Critical Developmental Window Has Lasting Metabolic Consequences. *Cell* **158**, 705–721 (2014).
 199. Gensollen, T., Iyer, S. S., Kasper, D. L. & Blumberg, R. S. How colonization by microbiota in early life shapes the immune system. *Science* **352**, 539–544 (2016).
 200. Metsälä, J. *et al.* Prenatal and post-natal exposure to antibiotics and risk of asthma in childhood. *Clin. Exp. Allergy* **45**, 137–145 (2015).
 201. Chu, S. *et al.* Periconceptional and Gestational Exposure to Antibiotics and Childhood Asthma. *PLOS ONE* **10**, e0140443 (2015).
 202. Russell, S. L. *et al.* Perinatal antibiotic treatment affects murine microbiota, immune responses and allergic asthma. *Gut Microbes* **4**, 158–164 (2013).
 203. Livanos, A. E. *et al.* Antibiotic-mediated gut microbiome perturbation accelerates development of type 1 diabetes in mice. *Nat. Microbiol.* **1**, 16140 (2016).
 204. Fåk, F., Ahrné, S., Molin, G., Jeppsson, B. & Weström, B. Microbial manipulation of the rat dam changes bacterial colonization and alters properties of the gut in her offspring. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **294**, G148–G154 (2008).
 205. O’Mahony, S. M. *et al.* Disturbance of the gut microbiota in early-life selectively affects visceral pain in adulthood without impacting cognitive or anxiety-related behaviors in male rats. *Neuroscience* **277**, 885–901 (2014).
 206. Dydensborg Sander, S. *et al.* Association Between Antibiotics in the First Year of Life and Celiac Disease. *Gastroenterology* **156**, 2217–2229 (2019).

207. Goethel, A. *et al.* Nod2 influences microbial resilience and susceptibility to colitis following antibiotic exposure. *Mucosal Immunol.* **12**, 720–732 (2019).
208. Poon, S. S. B. *et al.* Neonatal antibiotics have long term sex-dependent effects on the enteric nervous system. *J. Physiol.* **600**, 4303–4323 (2022).
209. Hung, L. Y. *et al.* Neonatal Antibiotics Disrupt Motility and Enteric Neural Circuits in Mouse Colon. *Cell. Mol. Gastroenterol. Hepatol.* **8**, 298-300.e6 (2019).
210. Chen, C.-M., Chou, H.-C. & Yang, Y.-C. S. H. Maternal Antibiotic Treatment Disrupts the Intestinal Microbiota and Intestinal Development in Neonatal Mice. *Front. Microbiol.* **12**, 684233 (2021).
211. Cho, I. *et al.* Antibiotics in early life alter the murine colonic microbiome and adiposity. *Nature* **488**, 621–626 (2012).
212. Chen, L.-W. *et al.* Implication of gut microbiota in the association between infant antibiotic exposure and childhood obesity and adiposity accumulation. *Int. J. Obes.* **44**, 1508–1520 (2020).
213. Lynn, M. A. *et al.* The composition of the gut microbiota following early-life antibiotic exposure affects host health and longevity in later life. *Cell Rep.* **36**, 109564 (2021).
214. Champagne-Jorgensen, K. *et al.* Prenatal low-dose penicillin results in long-term sex-specific changes to murine behaviour, immune regulation, and gut microbiota. *Brain. Behav. Immun.* **84**, 154–163 (2020).
215. Kayyal, M. *et al.* Sex dependent effects of post-natal penicillin on brain, behavior and immune regulation are prevented by concurrent probiotic treatment. *Sci. Rep.* **10**, 10318 (2020).
216. Leclercq, S. *et al.* Low-dose penicillin in early life induces long-term changes in murine gut microbiota, brain cytokines and behavior. *Nat. Commun.* **8**, 15062 (2017).
217. O'Connor, R. *et al.* Maternal antibiotic administration during a critical developmental window has enduring neurobehavioural effects in offspring mice. *Behav. Brain Res.* **404**, 113156 (2021).
218. Lynch, C. M. K. *et al.* Critical windows of early-life microbiota disruption on behaviour, neuroimmune function, and neurodevelopment. *Brain. Behav. Immun.* **108**, 309–327 (2023).
219. Madany, A. M., Hughes, H. K. & Ashwood, P. Antibiotic Treatment during Pregnancy Alters Offspring Gut Microbiota in a Sex-Dependent Manner. *Biomedicines* **10**, 1042 (2022).

220. Arentsen, T. *et al.* The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior. *Mol. Psychiatry* **22**, 257–266 (2017).
221. Cusick, S. E. & Georgieff, M. K. The Role of Nutrition in Brain Development: The Golden Opportunity of the “First 1000 Days”. *J. Pediatr.* **175**, 16–21 (2016).
222. Zeiss, C. J. Comparative Milestones in Rodent and Human Postnatal Central Nervous System Development. *Toxicol. Pathol.* **49**, 1368–1373 (2021).
223. Khodosevich, K. & Sellgren, C. M. Neurodevelopmental disorders—high-resolution rethinking of disease modeling. *Mol. Psychiatry* **28**, 34–43 (2023).
224. Dash, S., Syed, Y. A. & Khan, M. R. Understanding the Role of the Gut Microbiome in Brain Development and Its Association With Neurodevelopmental Psychiatric Disorders. *Front. Cell Dev. Biol.* **10**, 880544 (2022).
225. Codagnone, M. G. *et al.* Programming Bugs: Microbiota and the Developmental Origins of Brain Health and Disease. *Biol. Psychiatry* **85**, 150–163 (2019).
226. Götz, M., Nakafuku, M. & Petrik, D. Neurogenesis in the Developing and Adult Brain—Similarities and Key Differences. *Cold Spring Harb. Perspect. Biol.* **8**, a018853 (2016).
227. Humann, J. *et al.* Bacterial Peptidoglycan Traverses the Placenta to Induce Fetal Neuroproliferation and Aberrant Postnatal Behavior. *Cell Host Microbe* **19**, 388–399 (2016).
228. Vuong, H. E. *et al.* The maternal microbiome modulates fetal neurodevelopment in mice. *Nature* **586**, 281–286 (2020).
229. Oskvig, D. B., Elkahlon, A. G., Johnson, K. R., Phillips, T. M. & Herkenham, M. Maternal immune activation by LPS selectively alters specific gene expression profiles of interneuron migration and oxidative stress in the fetus without triggering a fetal immune response. *Brain. Behav. Immun.* **26**, 623–634 (2012).
230. Meckel, K. R. & Kiraly, D. D. Maternal microbes support fetal brain wiring. *Nature* **586**, 203–205 (2020).
231. Liu, G. *et al.* Gut dysbiosis impairs hippocampal plasticity and behaviors by remodeling serum metabolome. *Gut Microbes* **14**, 2104089 (2022).

232. Keogh, C. E. *et al.* Myelin as a regulator of development of the microbiota-gut-brain axis. *Brain. Behav. Immun.* **91**, 437–450 (2021).
233. Yamaguchi, Y. & Miura, M. Programmed Cell Death in Neurodevelopment. *Dev. Cell* **32**, 478–490 (2015).
234. Jena, A. *et al.* Gut-Brain Axis in the Early Postnatal Years of Life: A Developmental Perspective. *Front. Integr. Neurosci.* **14**, 44 (2020).
235. Luczynski, P. *et al.* Adult microbiota-deficient mice have distinct dendritic morphological changes: differential effects in the amygdala and hippocampus. *Eur. J. Neurosci.* **44**, 2654–2666 (2016).
236. Heijtz, R. D. *et al.* Normal gut microbiota modulates brain development and behavior. *Proc. Natl. Acad. Sci.* **108**, 3047–3052 (2011).
237. Arentsen, T., Raith, H., Qian, Y., Forssberg, H. & Heijtz, R. D. Host microbiota modulates development of social preference in mice. *Microb. Ecol. Health Dis.* **26**, (2015).
238. Barateiro, A., Brites, D. & Fernandes, A. Oligodendrocyte Development and Myelination in Neurodevelopment: Molecular Mechanisms in Health and Disease. *Curr. Pharm. Des.* **22**, 656–679 (2016).
239. Hoban, A. E. *et al.* Regulation of prefrontal cortex myelination by the microbiota. *Transl. Psychiatry* **6**, e774–e774 (2016).
240. Needham, B. D. *et al.* A gut-derived metabolite alters brain activity and anxiety behaviour in mice. *Nature* **602**, 647–653 (2022).
241. Gacias, M. *et al.* Microbiota-driven transcriptional changes in prefrontal cortex override genetic differences in social behavior. *eLife* **5**, e13442 (2016).
242. Daneman, R. & Prat, A. The Blood–Brain Barrier. *Cold Spring Harb. Perspect. Biol.* **7**, a020412 (2015).
243. Sun, N. *et al.* Antibiotic-induced microbiome depletion in adult mice disrupts blood-brain barrier and facilitates brain infiltration of monocytes after bone-marrow transplantation. *Brain. Behav. Immun.* **92**, 102–114 (2021).

244. Luck, B. *et al.* Bifidobacteria shape host neural circuits during postnatal development by promoting synapse formation and microglial function. *Sci. Rep.* **10**, 7737 (2020).
245. Thion, M. S. *et al.* Microbiome Influences Prenatal and Adult Microglia in a Sex-Specific Manner. *Cell* **172**, 500-516.e16 (2018).
246. Brown, D. G. *et al.* The microbiota protects from viral-induced neurologic damage through microglia-intrinsic TLR signaling. *eLife* **8**, e47117 (2019).
247. Jurek, B. & Neumann, I. D. The Oxytocin Receptor: From Intracellular Signaling to Behavior. *Physiol. Rev.* **98**, 1805–1908 (2018).
248. Grinevich, V. & Neumann, I. D. Brain oxytocin: how puzzle stones from animal studies translate into psychiatry. *Mol. Psychiatry* **26**, 265–279 (2021).
249. Grinevich, V., Knobloch-Bollmann, H. S., Eliava, M., Busnelli, M. & Chini, B. Assembling the Puzzle: Pathways of Oxytocin Signaling in the Brain. *Biol. Psychiatry* **79**, 155–164 (2016).
250. Grinevich, V., Desarménien, M. G., Chini, B., Tauber, M. & Muscatelli, F. Ontogenesis of oxytocin pathways in the mammalian brain: late maturation and psychosocial disorders. *Front. Neuroanat.* **8**, (2015).
251. Zhang, B. *et al.* Reconstruction of the Hypothalamo-Neurohypophysial System and Functional Dissection of Magnocellular Oxytocin Neurons in the Brain. *Neuron* **109**, 331-346.e7 (2021).
252. Knobloch, H. S. *et al.* Evoked Axonal Oxytocin Release in the Central Amygdala Attenuates Fear Response. *Neuron* **73**, 553–566 (2012).
253. Eliava, M. *et al.* A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing. *Neuron* **89**, 1291–1304 (2016).
254. Tang, Y. *et al.* Social touch promotes interfemale communication via activation of parvocellular oxytocin neurons. *Nat. Neurosci.* **23**, 1125–1137 (2020).
255. Grinevich, V., Desarménien, M. G., Chini, B., Tauber, M. & Muscatelli, F. Ontogenesis of oxytocin pathways in the mammalian brain: late maturation and psychosocial disorders. *Front. Neuroanat.* **8**, (2015).
256. Lewis, E. M. *et al.* Parallel Social Information Processing Circuits Are Differentially Impacted in Autism. *Neuron* **108**, 659-675.e6 (2020).

257. Carter, C. S. *et al.* Is Oxytocin “Nature’s Medicine”? *Pharmacol. Rev.* **72**, 829–861 (2020).
258. Báñez-López, S., Scanlon, L., Murphy, D. & Greenwood, M. P. Imaging the Hypothalamo-Neurohypophysial System. *Neuroendocrinology* **113**, 168–178 (2023).
259. Oti, T. *et al.* Oxytocin Influences Male Sexual Activity via Non-synaptic Axonal Release in the Spinal Cord. *Curr. Biol.* **31**, 103-114.e5 (2021).
260. Muscatelli, F., Desarménien, M. G., Matarazzo, V. & Grinevich, V. Oxytocin Signaling in the Early Life of Mammals: Link to Neurodevelopmental Disorders Associated with ASD. in *Behavioral Pharmacology of Neuropeptides: Oxytocin* (eds. Hurlemann, R. & Grinevich, V.) vol. 35 239–268 (Springer International Publishing, 2017).
261. Kompier, N. F., Keyzers, C., Gazzola, V., Lucassen, P. J. & Krugers, H. J. Early Life Adversity and Adult Social Behavior: Focus on Arginine Vasopressin and Oxytocin as Potential Mediators. *Front. Behav. Neurosci.* **13**, 143 (2019).
262. Caldwell, H. K. & Albers, H. E. Oxytocin, Vasopressin, and the Motivational Forces that Drive Social Behaviors. in *Behavioral Neuroscience of Motivation* (eds. Simpson, E. H. & Balsam, P. D.) vol. 27 51–103 (Springer International Publishing, 2015).
263. Otero-García, M., Agustín-Pavón, C., Lanuza, E. & Martínez-García, F. Distribution of oxytocin and co-localization with arginine vasopressin in the brain of mice. *Brain Struct. Funct.* **221**, 3445–3473 (2016).
264. Soumier, A., Habart, M., Lio, G., Demily, C. & Sirigu, A. Differential fate between oxytocin and vasopressin cells in the developing mouse brain. *iScience* **25**, 103655 (2022).
265. Madrigal, M. P. & Jurado, S. Specification of oxytocinergic and vasopressinergic circuits in the developing mouse brain. *Commun. Biol.* **4**, 586 (2021).
266. Son, S. *et al.* Whole-Brain Wiring Diagram of Oxytocin System in Adult Mice. *J. Neurosci.* **42**, 5021–5033 (2022).
267. Freda, S. N. *et al.* Brainwide input-output architecture of paraventricular oxytocin and vasopressin neurons. <http://biorxiv.org/lookup/doi/10.1101/2022.01.17.476652> (2022)
doi:10.1101/2022.01.17.476652.

268. Franklin, K. B. J. & Paxinos, G. *The mouse brain in stereotaxic coordinates*. (Academic Press, 1997).
269. Hammock, E. A. D. Developmental Perspectives on Oxytocin and Vasopressin. *Neuropsychopharmacology* **40**, 24–42 (2015).
270. Jing, X., Ratty, A. K. & Murphy, D. Ontogeny of the vasopressin and oxytocin RNAs in the mouse hypothalamus. *Neurosci. Res.* **30**, 343–349 (1998).
271. Almazan, G., Lefebvre, D. L. & Zingg, H. H. Ontogeny of hypothalamic vasopressin, oxytocin and somatostatin gene expression. *Dev. Brain Res.* **45**, 69–75 (1989).
272. Godefroy, D. *et al.* Three-dimensional distribution of tyrosine hydroxylase, vasopressin and oxytocin neurones in the transparent postnatal mouse brain. *J. Neuroendocrinol.* **29**, e12551 (2017).
273. Tamborski, S., Mintz, E. M. & Caldwell, H. K. Sex Differences in the Embryonic Development of the Central Oxytocin System in Mice. *J. Neuroendocrinol.* **28**, (2016).
274. Schaller, F. *et al.* A single postnatal injection of oxytocin rescues the lethal feeding behaviour in mouse newborns deficient for the imprinted *Magel2* gene. *Hum. Mol. Genet.* **19**, 4895–4905 (2010).
275. Chevalyere, V., Dayanithi, G., Moos, F. C. & Desarménien, M. G. Developmental Regulation of a Local Positive Autocontrol of Supraoptic Neurons. *J. Neurosci.* **20**, 5813–5819 (2000).
276. Chevalyere, V., Moos, F. C. & Desarménien, M. G. Correlation between electrophysiological and morphological characteristics during maturation of rat supraoptic neurons: Maturation of supraoptic neurons. *Eur. J. Neurosci.* **13**, 1136–1146 (2001).
277. Chevalyere, V., Moos, F. C. & Desarménien, M. G. Interplay between Presynaptic and Postsynaptic Activities Is Required for Dendritic Plasticity and Synaptogenesis in the Supraoptic Nucleus. *J. Neurosci.* **22**, 265–273 (2002).
278. Hammock, E. A. D. Oxytocin receptor ligand binding in embryonic tissue and postnatal brain development of the C57BL/6J mouse. *Front. Behav. Neurosci.* **7**, (2013).

279. Lukas, M., Bredewold, R., Neumann, I. D. & Veenema, A. H. Maternal separation interferes with developmental changes in brain vasopressin and oxytocin receptor binding in male rats. *Neuropharmacology* **58**, 78–87 (2010).
280. Zheng, J.-J. *et al.* Oxytocin mediates early experience–dependent cross-modal plasticity in the sensory cortices. *Nat. Neurosci.* **17**, 391–399 (2014).
281. Menon, R. *et al.* Oxytocin Signaling in the Lateral Septum Prevents Social Fear during Lactation. *Curr. Biol.* **28**, 1066–1078.e6 (2018).
282. Huber, D., Veinante, P. & Stoop, R. Vasopressin and Oxytocin Excite Distinct Neuronal Populations in the Central Amygdala. *Science* **308**, 245–248 (2005).
283. Viviani, D. *et al.* Oxytocin Selectively Gates Fear Responses Through Distinct Outputs from the Central Amygdala. *Science* **333**, 104–107 (2011).
284. Marlin, B. J., Mitre, M., D’amour, J. A., Chao, M. V. & Froemke, R. C. Oxytocin enables maternal behaviour by balancing cortical inhibition. *Nature* **520**, 499–504 (2015).
285. Mitre, M. *et al.* A Distributed Network for Social Cognition Enriched for Oxytocin Receptors. *J. Neurosci.* **36**, 2517–2535 (2016).
286. Dumais, K. M., Bredewold, R., Mayer, T. E. & Veenema, A. H. Sex differences in oxytocin receptor binding in forebrain regions: Correlations with social interest in brain region- and sex-specific ways. *Horm. Behav.* **64**, 693–701 (2013).
287. Han, M., Jiang, G., Luo, H. & Shao, Y. Neurobiological Bases of Social Networks. *Front. Psychol.* **12**, 626337 (2021).
288. Grinevich, V. & Ludwig, M. The multiple faces of the oxytocin and vasopressin systems in the brain. *J. Neuroendocrinol.* **33**, (2021).
289. Song, Z. & Albers, H. E. Cross-talk among oxytocin and arginine-vasopressin receptors: Relevance for basic and clinical studies of the brain and periphery. *Front. Neuroendocrinol.* **51**, 14–24 (2018).
290. Smith, C. J. W. *et al.* Age and sex differences in oxytocin and vasopressin V1a receptor binding densities in the rat brain: focus on the social decision-making network. *Brain Struct. Funct.* **222**, 981–1006 (2017).

291. Smith, C. J. W., DiBenedictis, B. T. & Veenema, A. H. Comparing vasopressin and oxytocin fiber and receptor density patterns in the social behavior neural network: Implications for cross-system signaling. *Front. Neuroendocrinol.* **53**, 100737 (2019).
292. Huber, D., Veinante, P. & Stoop, R. Vasopressin and Oxytocin Excite Distinct Neuronal Populations in the Central Amygdala. *Science* **308**, 245–248 (2005).
293. Hernández, V. S. *et al.* Hypothalamic Vasopressinergic Projections Innervate Central Amygdala GABAergic Neurons: Implications for Anxiety and Stress Coping. *Front. Neural Circuits* **10**, (2016).
294. Rood, B. D. & De Vries, G. J. Vasopressin innervation of the mouse (*Mus musculus*) brain and spinal cord. *J. Comp. Neurol.* **519**, 2434–2474 (2011).
295. Roper, J., O’Carroll, A.-M., Young, W. & Lolait, S. The vasopressin Avpr1b receptor: Molecular and pharmacological studies. *Stress* **14**, 98–115 (2011).
296. Corbani, M. *et al.* Neuroanatomical distribution and function of the vasopressin V1B receptor in the rat brain deciphered using specific fluorescent ligands. *Gen. Comp. Endocrinol.* **258**, 15–32 (2018).
297. Stevenson, E. L. & Caldwell, H. K. The vasopressin 1b receptor and the neural regulation of social behavior. *Horm. Behav.* **61**, 277–282 (2012).
298. DiBenedictis, B. T., Nussbaum, E. R., Cheung, H. K. & Veenema, A. H. Quantitative mapping reveals age and sex differences in vasopressin, but not oxytocin, immunoreactivity in the rat social behavior neural network: DIBENEDICTIS *et al.* *J. Comp. Neurol.* **525**, 2549–2570 (2017).
299. Viau, V. & Meaney, M. The inhibitory effect of testosterone on hypothalamic-pituitary-adrenal responses to stress is mediated by the medial preoptic area. *J. Neurosci.* **16**, 1866–1876 (1996).
300. Hasan, M. T. *et al.* A Fear Memory Engram and Its Plasticity in the Hypothalamic Oxytocin System. *Neuron* **103**, 133-146.e8 (2019).
301. Tribollet, E., Goumaz, M., Raggenbass, M., Dubois-Dauphin, M. & Dreifuss, J. J. Early appearance and transient expression of vasopressin receptors in the brain of rat fetus and infant. An autoradiographical and electrophysiological study. *Dev. Brain Res.* **58**, 13–24 (1991).

302. Hammock, E. A. D. & Levitt, P. Modulation of parvalbumin interneuron number by developmentally transient neocortical vasopressin receptor 1a (V1aR). *Neuroscience* **222**, 20–28 (2012).
303. Tribollet, E., Audigier, S., Dubois-Dauphin, M. & Dreifuss, J. J. Gonadal steroids regulate oxytocin receptors but not vasopressin receptors in the brain of male and female rats. An autoradiographical study. *Brain Res.* **511**, 129–140 (1990).
304. Nakajima, M., Görlich, A. & Heintz, N. Oxytocin Modulates Female Sociosexual Behavior through a Specific Class of Prefrontal Cortical Interneurons. *Cell* **159**, 295–305 (2014).
305. Li, K., Nakajima, M., Ibañez-Tallon, I. & Heintz, N. A Cortical Circuit for Sexually Dimorphic Oxytocin-Dependent Anxiety Behaviors. *Cell* **167**, 60-72.e11 (2016).
306. Peris, J. *et al.* Oxytocin receptors are expressed on dopamine and glutamate neurons in the mouse ventral tegmental area that project to nucleus accumbens and other mesolimbic targets: Oxytocin receptor expressing neurons in VTA. *J. Comp. Neurol.* **525**, 1094–1108 (2017).
307. Miyazaki, S., Hiraoka, Y., Hidema, S. & Nishimori, K. Prenatal minocycline treatment alters synaptic protein expression, and rescues reduced mother call rate in oxytocin receptor-knockout mice. *Biochem. Biophys. Res. Commun.* **472**, 319–323 (2016).
308. Loewen, S. P., Baimoukhametova, D. V. & Bains, J. S. Sex-Specific Vasopressin Signaling Buffers Stress-Dependent Synaptic Changes in Female Mice. *J. Neurosci.* **40**, 8842–8852 (2020).
309. Patisaul, H. B. Endocrine Disruption of Vasopressin Systems and Related Behaviors. *Front. Endocrinol.* **8**, 134 (2017).
310. Rigney, N., De Vries, G. J., Petrulis, A. & Young, L. J. Oxytocin, Vasopressin, and Social Behavior: From Neural Circuits to Clinical Opportunities. *Endocrinology* **163**, bqac111 (2022).
311. Rigney, N., De Vries, G. J. & Petrulis, A. Modulation of social behavior by distinct vasopressin sources. *Front. Endocrinol.* **14**, 1127792 (2023).
312. Bredewold, R. & Veenema, A. H. Sex differences in the regulation of social and anxiety-related behaviors: insights from vasopressin and oxytocin brain systems. *Curr. Opin. Neurobiol.* **49**, 132–140 (2018).

313. Engelmann, Ebner, Landgraf, Holsboer, & Wotjak. Emotional Stress Triggers Intrahypothalamic But Not Peripheral Release of Oxytocin in Male Rats: Dissociated central and peripheral release of oxytocin. *J. Neuroendocrinol.* **11**, 867–872 (2001).
314. Sabihi, S., Dong, S. M., Maurer, S. D., Post, C. & Leuner, B. Oxytocin in the medial prefrontal cortex attenuates anxiety: Anatomical and receptor specificity and mechanism of action. *Neuropharmacology* **125**, 1–12 (2017).
315. Leuner, B., Caponiti, J. M. & Gould, E. Oxytocin stimulates adult neurogenesis even under conditions of stress and elevated glucocorticoids. *Hippocampus* **22**, 861–868 (2012).
316. Duque-Wilckens, N. *et al.* Oxytocin Receptors in the Anteromedial Bed Nucleus of the Stria Terminalis Promote Stress-Induced Social Avoidance in Female California Mice. *Biol. Psychiatry* **83**, 203–213 (2018).
317. Whylings, J., Rigney, N., Peters, N. V., De Vries, G. J. & Petrulis, A. Sexually dimorphic role of BNST vasopressin cells in sickness and social behavior in male and female mice. *Brain. Behav. Immun.* **83**, 68–77 (2020).
318. Resendez, S. L. *et al.* Social Stimuli Induce Activation of Oxytocin Neurons Within the Paraventricular Nucleus of the Hypothalamus to Promote Social Behavior in Male Mice. *J. Neurosci.* **40**, 2282–2295 (2020).
319. Tan, Y. *et al.* Oxytocin Receptors Are Expressed by Glutamatergic Prefrontal Cortical Neurons That Selectively Modulate Social Recognition. *J. Neurosci.* **39**, 3249–3263 (2019).
320. Xiao, L., Priest, M. F., Nasenbeny, J., Lu, T. & Kozorovitskiy, Y. Biased Oxytocinergic Modulation of Midbrain Dopamine Systems. *Neuron* **95**, 368–384.e5 (2017).
321. Hörnberg, H. *et al.* Rescue of oxytocin response and social behaviour in a mouse model of autism. *Nature* **584**, 252–256 (2020).
322. Sala, M. *et al.* Pharmacologic Rescue of Impaired Cognitive Flexibility, Social Deficits, Increased Aggression, and Seizure Susceptibility in Oxytocin Receptor Null Mice: A Neurobehavioral Model of Autism. *Biol. Psychiatry* **69**, 875–882 (2011).

323. Lapp, H. E., Bartlett, A. A., Zup, S. L., Hunter, R. G. & Moore, C. L. Early experience alters developmental trajectory of central oxytocin systems involved in hypothalamic-pituitary-adrenal axis regulation in Long-Evans rats. *Horm. Behav.* **126**, 104822 (2020).
324. Howland, M. A., Sandman, C. A. & Glynn, L. M. Developmental origins of the human hypothalamic-pituitary-adrenal axis. *Expert Rev. Endocrinol. Metab.* **12**, 321–339 (2017).
325. Wamsteeker Cusulin, J. I., Füzesi, T., Watts, A. G. & Bains, J. S. Characterization of Corticotropin-Releasing Hormone neurons in the Paraventricular Nucleus of the Hypothalamus of Crh-IRES-Cre Mutant Mice. *PLoS ONE* **8**, e64943 (2013).
326. Sheng, J. A. *et al.* The Hypothalamic-Pituitary-Adrenal Axis: Development, Programming Actions of Hormones, and Maternal-Fetal Interactions. *Front. Behav. Neurosci.* **14**, 601939 (2021).
327. Dabrowska, J., Hazra, R., Guo, J.-D., DeWitt, S. & Rainnie, D. G. Central CRF neurons are not created equal: phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis. *Front. Neurosci.* **7**, (2013).
328. Viau, V., Bingham, B., Davis, J., Lee, P. & Wong, M. Gender and Puberty Interact on the Stress-Induced Activation of Parvocellular Neurosecretory Neurons and Corticotropin-Releasing Hormone Messenger Ribonucleic Acid Expression in the Rat. *Endocrinology* **146**, 137–146 (2005).
329. Aguilera, G. & Liu, Y. The molecular physiology of CRH neurons. *Front. Neuroendocrinol.* **33**, 67–84 (2012).
330. Van Pett, K. *et al.* Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J. Comp. Neurol.* **428**, 191–212 (2000).
331. Kühne, C. *et al.* Visualizing corticotropin-releasing hormone receptor type 1 expression and neuronal connectivities in the mouse using a novel multifunctional allele. *J. Comp. Neurol.* **520**, 3150–3180 (2012).
332. Müller, M. B. *et al.* Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. *Nat. Neurosci.* **6**, 1100–1107 (2003).
333. Refojo, D. *et al.* Glutamatergic and Dopaminergic Neurons Mediate Anxiogenic and Anxiolytic Effects of CRHR1. *Science* **333**, 1903–1907 (2011).

334. Patchev, V. The Neurosteroid Tetrahydroprogesterone Attenuates the Endocrine Response to Stress and Exerts Glucocorticoid-like Effects on Vasopressin Gene Transcription in the Rat Hypothalamus. *Neuropsychopharmacology* **15**, 533–540 (1996).
335. Grino, M., Young, W. S. & Burgunder, J.-M. Ontogeny of Expression of the Corticotropin-Releasing Factor Gene in the Hypothalamic Paraventricular Nucleus and of the Proopiomelanocortin Gene in Rat Pituitary. *Endocrinology* **124**, 60–68 (1989).
336. Gunn, B. G. *et al.* Dysfunctional Astrocytic and Synaptic Regulation of Hypothalamic Glutamatergic Transmission in a Mouse Model of Early-Life Adversity: Relevance to Neurosteroids and Programming of the Stress Response. *J. Neurosci.* **33**, 19534–19554 (2013).
337. Schmidt, M. *et al.* The postnatal development of the hypothalamic–pituitary–adrenal axis in the mouse. *Int. J. Dev. Neurosci.* **21**, 125–132 (2003).
338. Schlosser, S. F., Almeida, O. F., Patchev, V. K., Yassouridis, A. & Elands, J. Oxytocin-stimulated release of adrenocorticotropin from the rat pituitary is mediated by arginine vasopressin receptors of the V1b type. *Endocrinology* **135**, 2058–2063 (1994).
339. Sawchenko, P., Swanson, L. & Vale, W. Corticotropin-releasing factor: co-expression within distinct subsets of oxytocin-, vasopressin-, and neurotensin-immunoreactive neurons in the hypothalamus of the male rat. *J. Neurosci.* **4**, 1118–1129 (1984).
340. Neumann, I. D., Krömer, S. A., Toschi, N. & Ebner, K. Brain oxytocin inhibits the (re)activity of the hypothalamo–pituitary–adrenal axis in male rats: involvement of hypothalamic and limbic brain regions. *Regul. Pept.* **96**, 31–38 (2000).
341. Neumann, Wigger, Torner, Holsboer, & Landgraf. Brain Oxytocin Inhibits Basal and Stress-Induced Activity of the Hypothalamo-Pituitary-Adrenal Axis in Male and Female Rats: Partial Action Within the Paraventricular Nucleus: Effect of brain oxytocin on HPA axis of rats. *J. Neuroendocrinol.* **12**, 235–243 (2001).
342. Windle, R. J. *et al.* Oxytocin Attenuates Stress-Induced *c-fos* mRNA Expression in Specific Forebrain Regions Associated with Modulation of Hypothalamo–Pituitary–Adrenal Activity. *J. Neurosci.* **24**, 2974–2982 (2004).

343. Jurek, B. *et al.* Oxytocin Regulates Stress-Induced *Crf* Gene Transcription through CREB-Regulated Transcription Coactivator 3. *J. Neurosci.* **35**, 12248–12260 (2015).
344. Cohen, H. *et al.* Hippocampal microinfusion of oxytocin attenuates behavioral response to stress by means of dynamic interplay with the glucocorticoid-catecholamine responses. *J. Neuroendocrinol.* (2010) doi:10.1111/j.1365-2826.2010.02003.x.
345. Torner, L., Plotsky, P. M., Neumann, I. D. & De Jong, T. R. Forced swimming-induced oxytocin release into blood and brain: Effects of adrenalectomy and corticosterone treatment. *Psychoneuroendocrinology* **77**, 165–174 (2017).
346. Raadsheer, F. C., Sluiter, A. A., Ravid, R., Tilders, F. J. H. & Swaab, D. F. Localization of corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus of the human hypothalamus; age-dependent colocalization with vasopressin. *Brain Res.* **615**, 50–62 (1993).
347. Oyola, M. G., Thompson, M. K., Handa, A. Z. & Handa, R. J. Distribution and chemical composition of estrogen receptor β neurons in the paraventricular nucleus of the female and male mouse hypothalamus. *J. Comp. Neurol.* **525**, 3666–3682 (2017).
348. Johnson, K. V. A. & Burnet, P. W. J. Opposing effects of antibiotics and germ-free status on neuropeptide systems involved in social behaviour and pain regulation. *BMC Neurosci.* **21**, 32 (2020).
349. Effah, F. *et al.* Region-specific sex modulation of central oxytocin receptor by gut microbiota: An ontogenic study. *Dev. Neurobiol.* **81**, 149–163 (2021).
350. Kong, Q. *et al.* Daily intake of *Lactobacillus* alleviates autistic-like behaviors by ameliorating the 5-hydroxytryptamine metabolic disorder in VPA-treated rats during weaning and sexual maturation. *Food Funct.* **12**, 2591–2604 (2021).
351. Fields, C. T., Chassaing, B., Paul, M. J., Gewirtz, A. T. & De Vries, G. J. Vasopressin deletion is associated with sex-specific shifts in the gut microbiome. *Gut Microbes* **9**, 13–25 (2018).
352. Balázsfői, D. *et al.* Restoration of peripheral V2 receptor vasopressin signaling fails to correct behavioral changes in Brattleboro rats. *Psychoneuroendocrinology* **51**, 11–23 (2015).
353. Luo, Y. *et al.* Gut microbiota regulates mouse behaviors through glucocorticoid receptor pathway genes in the hippocampus. *Transl. Psychiatry* **8**, 187 (2018).

354. Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T. G. & Cryan, J. F. Microbiota is essential for social development in the mouse. *Mol. Psychiatry* **19**, 146–148 (2014).
355. Wu, W.-L. *et al.* Microbiota regulate social behaviour via stress response neurons in the brain. *Nature* **595**, 409–414 (2021).
356. Pusceddu, M. M. *et al.* Nod-like receptors are critical for gut–brain axis signalling in mice. *J. Physiol.* **597**, 5777–5797 (2019).
357. Lu, J. & Claud, E. C. Connection between gut microbiome and brain development in preterm infants. *Dev. Psychobiol.* **61**, 739–751 (2019).
358. Damiani, F., Cornuti, S. & Tognini, P. The gut-brain connection: Exploring the influence of the gut microbiota on neuroplasticity and neurodevelopmental disorders. *Neuropharmacology* **231**, 109491 (2023).
359. Bojović, K. *et al.* Gut Microbiota Dysbiosis Associated With Altered Production of Short Chain Fatty Acids in Children With Neurodevelopmental Disorders. *Front. Cell. Infect. Microbiol.* **10**, 223 (2020).
360. Bezawada, N., Phang, T. H., Hold, G. L. & Hansen, R. Autism Spectrum Disorder and the Gut Microbiota in Children: A Systematic Review. *Ann. Nutr. Metab.* **76**, 16–29 (2020).
361. Liu, J. *et al.* Alteration of Gut Microbiota: New Strategy for Treating Autism Spectrum Disorder. *Front. Cell Dev. Biol.* **10**, 792490 (2022).
362. Neuman, H., Forsythe, P., Uzan, A., Avni, O. & Koren, O. Antibiotics in early life: dysbiosis and the damage done. *FEMS Microbiol. Rev.* (2018) doi:10.1093/femsre/fuy018.
363. Hussey, S. *et al.* Parenteral Antibiotics Reduce Bifidobacteria Colonization and Diversity in Neonates. *Int. J. Microbiol.* **2011**, 1–6 (2011).
364. Aversa, Z. *et al.* Association of Infant Antibiotic Exposure With Childhood Health Outcomes. *Mayo Clin. Proc.* **96**, 66–77 (2021).
365. Axelsson, P. B. *et al.* Relation Between Infant Microbiota and Autism?: Results from a National Cohort Sibling Design Study. *Epidemiology* **30**, 52–60 (2019).

366. Kenyon, S. *et al.* Childhood outcomes after prescription of antibiotics to pregnant women with spontaneous preterm labour: 7-year follow-up of the ORACLE II trial. *The Lancet* **372**, 1319–1327 (2008).
367. Njotto, L. L. *et al.* Maternal and Early-Life Exposure to Antibiotics and the Risk of Autism and Attention-Deficit Hyperactivity Disorder in Childhood: a Swedish Population-Based Cohort Study. *Drug Saf.* **46**, 467–478 (2023).
368. Lavebratt, C. *et al.* Early exposure to antibiotic drugs and risk for psychiatric disorders: a population-based study. *Transl. Psychiatry* **9**, 317 (2019).
369. Slykerman, R. F. *et al.* Antibiotics in the first year of life and subsequent neurocognitive outcomes. *Acta Paediatr.* **106**, 87–94 (2017).
370. Slykerman, R. F. *et al.* Exposure to antibiotics in the first 24 months of life and neurocognitive outcomes at 11 years of age. *Psychopharmacology (Berl.)* **236**, 1573–1582 (2019).
371. Slob, E. M. A. *et al.* Early-life antibiotic use and risk of attention-deficit hyperactivity disorder and autism spectrum disorder: results of a discordant twin study. *Int. J. Epidemiol.* **50**, 475–484 (2021).
372. Firestein, M. R. *et al.* Perinatal antibiotics alter preterm infant EEG and neurobehavior in the Family Nurture Intervention trial. *Dev. Psychobiol.* **61**, 661–669 (2019).
373. Duong, Q. A., Pittet, L. F., Curtis, N. & Zimmermann, P. Antibiotic exposure and adverse long-term health outcomes in children: A systematic review and meta-analysis. *J. Infect.* **85**, 213–300 (2022).
374. Yu, H., Zhou, Y., Pan, L., Zhang, X. & Jiang, H. Early Life Antibiotic Exposure and the Subsequent Risk of Autism Spectrum Disorder and Attention Deficit Hyperactivity Disorder: A Systematic Review and Meta-Analysis. *J. Autism Dev. Disord.* **52**, 2236–2246 (2022).
375. Tochitani, S. *et al.* Administration of Non-Absorbable Antibiotics to Pregnant Mice to Perturb the Maternal Gut Microbiota Is Associated with Alterations in Offspring Behavior. *PLOS ONE* **11**, e0138293 (2016).
376. Lach, G. *et al.* Enduring neurobehavioral effects induced by microbiota depletion during the adolescent period. *Transl. Psychiatry* **10**, 382 (2020).

377. Sampson, T. R. *et al.* Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell* **167**, 1469-1480.e12 (2016).
378. Guida, F. *et al.* Antibiotic-induced microbiota perturbation causes gut endocannabinoidome changes, hippocampal neuroglial reorganization and depression in mice. *Brain. Behav. Immun.* **67**, 230–245 (2018).
379. Fröhlich, E. E. *et al.* Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. *Brain. Behav. Immun.* **56**, 140–155 (2016).
380. Degroote, S., Hunting, D. J., Baccarelli, A. A. & Takser, L. Maternal gut and fetal brain connection: Increased anxiety and reduced social interactions in Wistar rat offspring following periconceptional antibiotic exposure. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **71**, 76–82 (2016).
381. Hoban, A. E. *et al.* Microbial regulation of microRNA expression in the amygdala and prefrontal cortex. *Microbiome* **5**, 102 (2017).
382. Hoban, A. E. *et al.* Behavioural and neurochemical consequences of chronic gut microbiota depletion during adulthood in the rat. *Neuroscience* **339**, 463–477 (2016).
383. Van De Wouw, M., Boehme, M., Dinan, T. G. & Cryan, J. F. Monocyte mobilisation, microbiota & mental illness. *Brain. Behav. Immun.* **81**, 74–91 (2019).
384. Wu, Q. *et al.* Gut microbiota modulates stress-induced hypertension through the HPA axis. *Brain Res. Bull.* **162**, 49–58 (2020).
385. Iglesias-Vázquez, L., Van Ginkel Riba, G., Arijia, V. & Canals, J. Composition of Gut Microbiota in Children with Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. *Nutrients* **12**, 792 (2020).
386. Xu, M., Xu, X., Li, J. & Li, F. Association Between Gut Microbiota and Autism Spectrum Disorder: A Systematic Review and Meta-Analysis. *Front. Psychiatry* **10**, 473 (2019).
387. Golubeva, A. V. *et al.* Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. *EBioMedicine* **24**, 166–178 (2017).

388. Lin, T.-L. *et al.* Amelioration of Maternal Immune Activation-Induced Autism Relevant Behaviors by Gut Commensal Parabacteroides goldsteinii. *Int. J. Mol. Sci.* **23**, 13070 (2022).
389. Strandwitz, P. *et al.* GABA-modulating bacteria of the human gut microbiota. *Nat. Microbiol.* **4**, 396–403 (2018).
390. Ding, Y. *et al.* A next-generation probiotic: Akkermansia muciniphila ameliorates chronic stress-induced depressive-like behavior in mice by regulating gut microbiota and metabolites. *Appl. Microbiol. Biotechnol.* **105**, 8411–8426 (2021).
391. Sun, Y., Zhu, H., Cheng, R., Tang, Z. & Zhang, M. Outer membrane protein Amuc_1100 of Akkermansia muciniphila alleviates antibiotic-induced anxiety and depression-like behavior in mice. *Physiol. Behav.* **258**, 114023 (2023).
392. Yang, Y. *et al.* Early-life high-fat diet-induced obesity programs hippocampal development and cognitive functions via regulation of gut commensal Akkermansia muciniphila. *Neuropsychopharmacology* **44**, 2054–2064 (2019).
393. Lebovitz, Y. *et al.* Lactobacillus rescues postnatal neurobehavioral and microglial dysfunction in a model of maternal microbiome dysbiosis. *Brain. Behav. Immun.* **81**, 617–629 (2019).
394. Coretti, L. *et al.* Sex-related alterations of gut microbiota composition in the BTBR mouse model of autism spectrum disorder. *Sci. Rep.* **7**, 45356 (2017).
395. El-Ansary, A., Bhat, R. S. & Zayed, N. Gut Microbiome and Sex Bias in Autism Spectrum Disorders. *Curr. Behav. Neurosci. Rep.* **7**, 22–31 (2020).
396. Jang, H.-M., Lee, H.-J., Jang, S.-E., Han, M. J. & Kim, D.-H. Evidence for interplay among antibacterial-induced gut microbiota disturbance, neuro-inflammation, and anxiety in mice. *Mucosal Immunol.* **11**, 1386–1397 (2018).
397. Moya-Pérez, A., Perez-Villalba, A., Benítez-Páez, A., Campillo, I. & Sanz, Y. Bifidobacterium CECT 7765 modulates early stress-induced immune, neuroendocrine and behavioral alterations in mice. *Brain. Behav. Immun.* **65**, 43–56 (2017).
398. Korpela, K. *et al.* Probiotic supplementation restores normal microbiota composition and function in antibiotic-treated and in caesarean-born infants. *Microbiome* **6**, 182 (2018).

399. Luoto, R., Isolauri, E. & Lehtonen, L. Safety of *Lactobacillus* GG Probiotic in Infants with Very Low Birth Weight: Twelve Years of Experience. *Clin. Infect. Dis.* **50**, 1327–1328 (2010).
400. Luoto, R. *et al.* Prebiotic and probiotic supplementation prevents rhinovirus infections in preterm infants: A randomized, placebo-controlled trial. *J. Allergy Clin. Immunol.* **133**, 405–413 (2014).
401. Buffington, S. A. *et al.* Dissecting the contribution of host genetics and the microbiome in complex behaviors. *Cell* **184**, 1740-1756.e16 (2021).
402. Luck, B. *et al.* Bifidobacteria shape host neural circuits during postnatal development by promoting synapse formation and microglial function. *Sci. Rep.* **10**, 7737 (2020).
403. Guang, S. *et al.* Synaptopathology Involved in Autism Spectrum Disorder. *Front. Cell. Neurosci.* **12**, 470 (2018).
404. Sen, P. *et al.* The live biotherapeutic *Blautia stercoris* MRx0006 attenuates social deficits, repetitive behaviour, and anxiety-like behaviour in a mouse model relevant to autism. *Brain. Behav. Immun.* **106**, 115–126 (2022).
405. Dinan, K. & Dinan, T. Antibiotics and mental health: The good, the bad and the ugly. *J. Intern. Med.* **292**, 858–869 (2022).
406. Nazarian, S. & Akhondi, H. *Minocycline*. (StatPearls Publishing LLC, 2022).
407. Kumar, H. & Sharma, B. Minocycline ameliorates prenatal valproic acid induced autistic behaviour, biochemistry and blood brain barrier impairments in rats. *Brain Res.* **1630**, 83–97 (2016).
408. Toledo, M. A., Wen, T. H., Binder, D. K., Ethell, I. M. & Razak, K. A. Reversal of ultrasonic vocalization deficits in a mouse model of Fragile X Syndrome with minocycline treatment or genetic reduction of MMP-9. *Behav. Brain Res.* **372**, 112068 (2019).
409. Luo, Y. *et al.* Minocycline improves autism-related behaviors by modulating microglia polarization in a mouse model of autism. *Int. Immunopharmacol.* **122**, 110594 (2023).
410. Schmidtner, A. K. *et al.* Minocycline alters behavior, microglia and the gut microbiome in a trait-anxiety-dependent manner. *Transl. Psychiatry* **9**, 223 (2019).

411. Dooling, S. W., Sgritta, M., Wang, I.-C., Duque, A. L. R. F. & Costa-Mattioli, M. The Effect of *Limosilactobacillus reuteri* on Social Behavior Is Independent of the Adaptive Immune System. *mSystems* **7**, e00358-22 (2022).
412. Zuffa, S. *et al.* Early-life differences in the gut microbiota composition and functionality of infants at elevated likelihood of developing autism spectrum disorder. *Transl. Psychiatry* **13**, 257 (2023).
413. Swann, J. R., Spitzer, S. O. & Diaz Heijtz, R. Developmental Signatures of Microbiota-Derived Metabolites in the Mouse Brain. *Metabolites* **10**, 172 (2020).
414. Dan, B. Sex differences in neurodevelopmental disorders. *Dev. Med. Child Neurol.* **63**, 492–492 (2021).
415. Bastos, P. A. D., Wheeler, R. & Boneca, I. G. Uptake, recognition and responses to peptidoglycan in the mammalian host. *FEMS Microbiol. Rev.* **45**, fuaa044 (2021).
416. Wheeler, R., Chevalier, G., Eberl, G. & Gomperts Boneca, I. The biology of bacterial peptidoglycans and their impact on host immunity and physiology: Bacterial peptidoglycans in the mammalian host. *Cell. Microbiol.* **16**, 1014–1023 (2014).
417. Irazoki, O., Hernandez, S. B. & Cava, F. Peptidoglycan Muropeptides: Release, Perception, and Functions as Signaling Molecules. *Front. Microbiol.* **10**, 500 (2019).
418. Pandey, N. & Cascella, M. *Beta-Lactam Antibiotics*. (StatPearls Publishing LLC, 2023).
419. Tan, C. T., Xu, X., Qiao, Y. & Wang, Y. A peptidoglycan storm caused by β -lactam antibiotic's action on host microbiota drives *Candida albicans* infection. *Nat. Commun.* **12**, 2560 (2021).
420. Tosoni, G., Conti, M. & Diaz Heijtz, R. Bacterial peptidoglycans as novel signaling molecules from microbiota to brain. *Curr. Opin. Pharmacol.* **48**, 107–113 (2019).
421. Clarke, T. B. *et al.* Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* **16**, 228–231 (2010).
422. Al Nabhani, Z., Dietrich, G., Hugot, J.-P. & Barreau, F. Nod2: The intestinal gate keeper. *PLOS Pathog.* **13**, e1006177 (2017).
423. Petnicki-Ocwieja, T. *et al.* Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc. Natl. Acad. Sci.* **106**, 15813–15818 (2009).

424. Gabanyi, I. *et al.* Bacterial sensing via neuronal Nod2 regulates appetite and body temperature. *Science* **376**, eabj3986 (2022).
425. Arentsen, T., Khalid, R., Qian, Y. & Diaz Heijtz, R. Sex-dependent alterations in motor and anxiety-like behavior of aged bacterial peptidoglycan sensing molecule 2 knockout mice. *Brain. Behav. Immun.* **67**, 345–354 (2018).
426. Wang, Y., Liu, X. & Liu, Q. NOD2 Expression in *Streptococcus pneumoniae* Meningitis and Its Influence on the Blood-Brain Barrier. *Can. J. Infect. Dis. Med. Microbiol.* **2018**, 1–8 (2018).

Abstract

The gut microbiota – the trillions of microorganisms inhabiting the gastrointestinal tract, including bacteria, yeasts and viruses – is increasingly recognized as a key environmental factor that shapes host development and physiology. Over the last two decades, the gut microbiota has been identified as an essential component of the gut-brain axis, leading to the emerging concept of the "Microbiota-Gut-Brain axis". Preclinical studies using germ-free animals have demonstrated that the gut microbiota influences a wide range of neurodevelopmental processes including, the maturation and function of microglia, primary resident immune cells of the brain, blood brain barrier (BBB) integrity, and myelination. Moreover, several epidemiological studies suggest that broad-spectrum antibiotic exposure around the time of birth could have detrimental health outcomes, including childhood behavioral and emotional issues. However, it is unclear whether antibiotic exposure during a narrow perinatal window could influence neurodevelopmental outcomes. In this context, the aim of this thesis was to investigate the impact of maternal antibiotic exposure during a narrow critical perinatal window (*i.e.*, last week of pregnancy and first three postnatal days) on physiological and neurobehavioral outcomes of the offspring, including the development of socio-emotional behaviors and the expression of genes associated with intestinal barrier function, BBB integrity and the oxytocin and vasopressin receptor systems. Our results demonstrate that neonatal offspring from antibiotic-treated dams display an altered pattern of ultrasonic communication, which was more pronounced in males. Moreover, juvenile offspring from antibiotic-treated dams showed reduced social motivation and interaction, as well as context-dependent anxiety-like behavior. In addition, the juvenile offspring from exposed dams also displayed an altered gut microbiota composition. However, no changes were observed in locomotor or exploratory activity. This behavioral phenotype is strongly male-biased and is associated with BBB impairments and a mild colonic inflammatory response. Strikingly, perinatal ampicillin exposure leads to reduced gene expression of the oxytocin receptor in the prefrontal cortex of both male and female juvenile offspring and increased gene expression of the vasopressin receptor 1a in the amygdala of the female offspring, which are key regions involved in the regulation of socio-emotional behaviors. Altogether, these findings highlight the importance of the maternal microbiota in early-life, and how its perturbation by a widely used antibiotic could contribute to atypical social and emotional development of offspring in a sex-dependent manner.

Key words: Perinatal; Antibiotics; Gut microbiota; Socio-emotional behaviors; Neuropeptidergic systems.