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L'accouchement par césarienne à terme ou pré-terme n'induit pas de conséquences néfastes à long terme chez la souris

Term or preterm cesarean section delivery does not lead to long-term detrimental consequences in mice

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Soutenue le 27 juin 2018 devant le jury composé de

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RESUME

Les études épidémiologiques fournissent des données contradictoires sur les séquelles néfastes de la naissance par césarienne et leurs liens avec des troubles neurodéveloppementaux tels que l'autisme (TSA). En effet, les prévalences des césariennes et des TSA ont parallèlement augmenté ces dernières années. Pour avoir un meilleur aperçu du problème, mon travail de thèse a consisté à comprendre si la césarienne, à terme ou pré-terme, avait une influence sur l'apparition des TSA. Par conséquent, nous avons mis en place un modèle de césarienne à terme ou pré-terme chez la souris et évalué en conditions contrôlées des paramètres clés de leur comportement ainsi que de leur développement morphologique et physiologique.

Nos résultats ont montré que la césarienne à terme ou pré-terme n'altère pas les comportements sociaux, anxieux et locomoteurs des souris adultes. De plus, nous avons confirmé que la césarienne n'altère pas l'action du GABA qui passe d'excitatrice à inhibitrice chez les souris pendant leur seconde semaine postnatale. Par ailleurs, quelle que soit la manière d'accoucher, les courants postsynaptiques glutamatergiques et GABAergiques ont une amplitude et une fréquence similaires. De ce fait, la césarienne à terme ou pré-terme n'induit pas de conséquences préjudiciables à long terme telles que les activités physiologiques et comportements de type autistique. Cependant, nous avons constaté que les vocalisations ultrasoniques induites par l'isolation sont altérées pendant le développement chez les souriceaux nés par césarienne pré-terme. Ces résultats nous ont suggéré que la césarienne pourrait affecter d'autres paramètres développementaux plus précocement. Nos résultats ont montré qu'à la naissance, chez les souris nées par césarienne à terme, et encore plus chez les pré-terme, les neurones pyramidaux de CA3 présentent une arborisation dendritique sous-développée. Néanmoins, ces différences sont transitoires car un jour plus tard, leur arborisation dendritique neuronale est semblable à celle des animaux nés par voie basse. Globalement, ces résultats suggèrent que la césarienne, avec la prématurité comme facteur aggravant, induit des retards éphémères mais pas de conséquences à long terme.

En conclusion, ce travail de thèse suggère que la naissance par césarienne n'est pas suffisante pour induire des conséquences néfastes à long terme, comme indiqué par les études épidémiologiques.

SUMMARY

Epidemiological studies have provided contradictory data on the deleterious sequels of birth by cesarean section (C-section) delivery and their links with developmental brain disorders such as Autism Spectrum Disorders (ASD). To gain better insight on this issue, my dissertation work aimed to understand if birth by C-section (either at term or preterm) had an influence on the prevalence of ASD, both of which have increased in recent years. Hence, we implemented a mouse model of C-section delivery at term or preterm and evaluated in well-controlled conditions several key features of morphological and physiological development as well as behavior.

Our results show that in young-adult mice, C-section delivery at term or preterm did not alter social, anxious or locomotor behaviors. Furthermore, we confirmed that C-section delivery did not induce autistic-like features by assessing the GABA developmental excitatory to inhibitory shift. We found that, in two-week-old mice born either vaginally or by C-section delivery, GABA had an inhibitory action in CA3 pyramidal neurons. Moreover, GABAergic and glutamatergic postsynaptic currents had similar amplitude and frequency in vaginally-born and C-section delivered pups. Therefore, C-section delivery at term or preterm does not lead to long-term detrimental consequences such as ASD-like features and behaviors. Yet, we found that isolation-induced ultrasonic vocalizations were affected during the development of pups delivered preterm by C-section, raising the question that C-section delivery might still affect early neonatal developmental patterns. Our results showed that at birth, CA3 pyramidal neurons of pups born by C-section delivery at term, and even more at preterm, presented an underdeveloped arbor. Still, these differences were transient since one day later these neurons' morphology was similar to the one of pups delivered vaginally. Altogether, these results suggest that C-section delivery, with prematurity as an aggravating factor, induces transient developmental delays that do not lead to long-term consequences.

Overall, this dissertation work proposes that C-section delivery alone is not sufficient to induce long-term detrimental consequences as reported in epidemiological studies.

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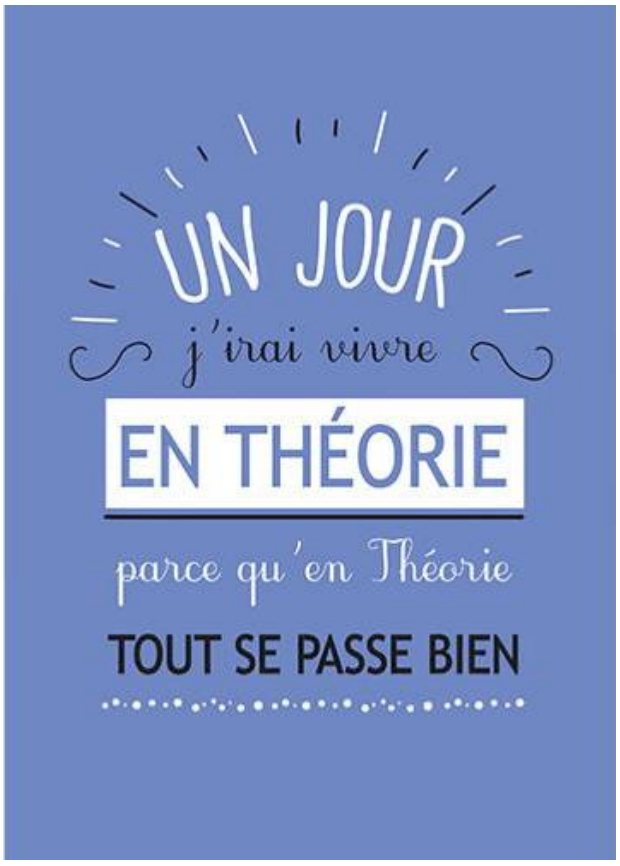

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15α-OH-DHEA: 15α-hydroxydehydroepiandrosterone

15α-OH-DHEA-S: 15α-hydroxydehydroepiandrosterone sulfate

16α-OH-DHEA: 16α-hydroxydehydroepiandrosterone

16α-OH-DHEA-S: 16α-hydroxydehydroepiandrosterone sulfate

17β-HSD: 17β-hydroxysteroid dehydrogenase enzyme

17-OH-progesterone: 17α-hydroxyprogesterone

3β-HSD: 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase enzyme

5-HT: Serotonin

5-HT_{1A}: Serotonin receptor subtype 1A

5-HT_{2A}: Serotonin receptor subtype 2A

A

AA: Arachidonic acid

AC: Adenylate cyclase

ACTH: Adrenocorticotropic hormone

ADHD: Attention-deficit hyperactivity disorder

AM: Amygdala

AMPH: Amphetamine

AON: Anterior olfactory nucleus

ASD: Autism spectrum disorders

ASMT: Acetylserotonin O-methyltransferase

ATP: Adenosine triphosphate

B

BD: Bipolar disorder

BDNF: Brain-derived neurotrophic factor

bFGF: Basic fibroblast growth factor

C

C-section: Cesarean section delivery

CA: Cornu Ammonis

CaM: Calmodulin

cAMP: Cyclic adenosine monophosphate

CCK: Cholecystokinin

Cg cx: Cingulate cortex

CGE: Caudal ganglionic eminence

CNS: Central nervous system

COX-2: Cyclooxygenase-2

cPLA$_2$: Cytosolic phospholipase A$_2$

CRF: Corticotropin releasing factor

CRH: Corticotropin releasing hormone

CTD: Chronic tic disorder

CX-43: Connexin 43

D

DAG: Diacylglycerol

DAT: Dopamine transporter

DG: Dentate gyrus

DHEA: Dehydroepiandrosterone

DHEA-S: Dehydroepiandrosterone sulfate

Drd1: Dopamine receptor subtype 1

Drd2: Dopamine receptor subtype 2

DSM-V: Diagnostic and statistical manual of mental disorders 5th edition

E

EC: Entorhinal cortex

EP1: Prostaglandin E subtype receptor 1

EP2: Prostaglandin E subtype receptor 2

EP3: Prostaglandin E subtype receptor 3

ER: Estrogens receptor

ERα: Estrogens receptor subtype α

ERβ: Estrogens receptor subtype β

G

GABA: γ-aminobutyric acid

GAD: Glutamic acid decarboxylase

GDP: Giant depolarizing potentials

GIRK: G protein-coupled inwardly-rectifying potassium channel

GM: Gray matter

HPA axis: Hypothalamo-pituitary-adrenal axis

HPC: Hippocampus

I

ICD-10: 10th revision of the International statistical classification of diseases and related health problems

IL cx: Infralimbic cortex

IP3: Inositol 1,4,5-triphosphate

IZ: Intermediated zone

K-L

KCC2: K^+-Cl^- co-transporter type 2

LM: Lacunosum moleculare

M

M1: Muscarinic receptor subtype 1

M2: Muscarinic receptor subtype 2

MAPK: Mitogen-activated protein kinases

MAZ: Multipolar cell accumulation zone

MGE: Medial ganglionic eminence

mGluR: Glutamate metabotropic receptor

MHC: Myosin heavy chains

MIA: Maternal immune activation

MLC_{17}: Myosin essential light chains

MLC_{20}: Myosin regulatory light chains

MLCK: Myosin light-chain kinase

MolCm: molecular cell layer of the cerebellum

MRI: Magnetic resonance imaging

mTOR: Mammalian target of rapamycin

MZ: Marginal zone

N

Nacc: Nucleus accumbens

nAchR: Nicotinic acetylcholine receptor

NAS: N-acetylserotonin

NKCC1: Na^+-K^+-Cl^- co-transporter type 1

NPY: Neuropeptide Y

NO: Nitric oxide

O

OCD: Obsessive-compulsive disorder

O-LM: Stratum oriens-lacunosum moleculare

OT: Oxytocin

OTb: Olfactory tubercle

OTR: Oxytocin receptor

P

P-450_{arom}: Aromatase enzymes

P-450_{c15}: 15α-hydroxylase enzyme

P-450_{c16}: 16α-hydroxylase enzyme

P-450_{c17}: 17α-hydroxylase enzyme

P-450_{scc}: P-450 side-chain cleavage enzyme

PBF: Pulmonary blood flow

PCO_2: Partial pressure of carbon dioxide

PDD: Pervasive developmental disorder

PEPCK: Phosphoenolpyruvate carboxykinase

PFC: Prefrontal cortex

PGD2: Prostaglandin D

PGD synthase: Prostaglandin D synthase

PGE2: Prostaglandin E

PGE synthase: Prostaglandin E synthase

$PGF_{2\alpha}$: Prostaglandin F

PGF synthase: Prostaglandin F synthase

PGH2: Prostaglandin H

PGI_2: Prostaglandin I_2

PIP2: Phosphatidylinositol 4,5-biphosphate

PKC: Protein kinase C

PLA_2: Phospholipase A_2

PLC: Phospholipase C

PO_2: Partial pressure of oxygen

PR: Progesterone receptor

PRA: Progesterone receptor subtype A

PRB: Progesterone receptor subtype B

PV: Parvalbumin

PVR: Pulmonary vascular resistance

R

rT3: Reverse tri-iodothyronine

RVO: Right ventricular output

S

SERT: Serotonin transporter

SNc: Substantia nigra pars compacta

SOM: Somatostatin

SPA: Synchronous plateau assemblies

Str: Striatum

SVZ : Subventricular zone

T-U

T3: Tri-iodothyronine

T4: Thyroxine

TDM: Tertiary dentate matrix

Th: Thalamus

TS: Tourette syndrome

TSH: Thyroid stimulating hormones

UCP1: Uncoupling protein 1

V-W

VGAT: Vesicular GABA transporter

VH: Ventral hippocampus

VIP: Vasoactive intestinal peptide

VPA: Valproic acid

VTA: Ventral tegmental area

VZ: Ventricular zone

WM: White matter

INTRODUCTION

1. PARTURITION

Delivery is amongst the most complex biological processes in mammals. Adjustments which rely on mechanical and hormonal factors must take place in a short period of time to allow the adaptation of the fetus to its external environment. These seemingly independent adjustments are actually integrated physiological events that can be grouped in three categories: initiation of parturition, active labor, and fetal adaptation from intra- to extrauterine life.

Prior to studying the deleterious effects that might be induced by a perinatal affection, we must first understand the physiological processes which lead to the fetus' autonomous development.

1.1. Initiation of parturition in mammals

1.1.1. Placenta and fetal membranes

Fetal membranes are metabolically active membranes which surround and protect the developing fetus. They consist of the amnion, the chorion, the yolk sac, and the allantois[1]. Throughout pregnancy, they isolate the amniotic fluid from the maternal circulation and their rupture marks the termination of pregnancy.

The placenta is a temporary feto-maternal organ composed of the fetal chorion and maternal decidua (derived from the uterus lining) that allows the transfer of oxygen and nutrients from the mother to the fetus, and the release of carbon dioxide and waste products from the fetus[2].

Altogether, these structures regulate gestation and trigger the beginning of parturition by releasing four main molecules: progesterone, estrogen, prostaglandins and corticotropin releasing hormone.

1.1.1.1. Progesterone

Progesterone is an endogenous steroid hormone that is essential for the maintenance of gestation. In early stages (prior to 10 weeks of gestation), progesterone, which mainly comes from the corpus luteum, suppresses the maternal immune response to fetal antigens, hence preventing maternal rejection of the trophoblast[3].

By the tenth week of gestation, progesterone production switches from the corpus luteum to the placenta[4] (Figure 1) in humans. In this structure, the P-450 side-chain cleavage enzyme (P450-

scc) converts maternal cholesterol into pregnenolone which, in turn, is converted by the 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase enzyme (3β-HSD) into progesterone. Due to the low placental 17α-hydroxylase (P-450$_{c17}$) activity at the early stages of gestation, progesterone is not further converted into androgens. Thus, progesterone levels efficiently rise throughout pregnancy until parturition. In the myometrium (middle layer of the uterine wall responsible for contractions), the maintenance of high progesterone levels results in the (1) inhibition of the gap junction formation required for uterine contractility, (2) facilitation of β-adrenergic receptor expression (upon which catecholamines stimulation induces uterine relaxation), and (3) reduced myometrial sensitivity to labor-inducing agents during gestation[5].

In most subprimate mammals, an essential step for parturition to occur is the abrupt drop in maternal plasma progesterone levels before the onset of labor[6–8]. This drop is caused by a change in placental steroidogenesis in favor of androgens production. In fact, fetal adrenal cortisol, whose production sharply increases at term, induces the conversion of progesterone into androgens by indirectly stimulating P-450$_{c17}$ expression, hence decreasing progesterone placental levels. In pregnant goats, absence of progesterone withdrawal from the myometrium results in a massive rise in estrogens in an attempt to terminate pregnancy[9].

Contrary to these species, in humans, progesterone production by the placenta does not abruptly drop before the onset of birth[10–12]. Nonetheless, in healthy women that gave birth at term, a steady and non-abrupt decrease is observed starting from the 36th week of gestation[13]. The absence of progesterone sudden withdrawal may be due to the fact that, in humans, P-450$_{c17}$ expression is not dependent on cortisol levels. However, a progesterone-binding protein has been detected in the chorion during the last weeks of gestation. During late gestation, an increase in this protein level has been reported which may result in a local withdrawal of progesterone level that cannot be detected in the peripheral plasma[14]. This hypothesis is further supported by the evidence of decreased progesterone metabolism in the chorioamnion when the amount of binding protein increases[15].

1.1.1.2. Estrogens

Estrogens are endogenous steroid hormones which are required for promoting uterine contractility during active labor and parturition. Four major isoforms are found in humans: estrone, estradiol, estriol and estetrol. Similar to progesterone, estrogens are synthetized in the placenta during pregnancy (Figure 1).

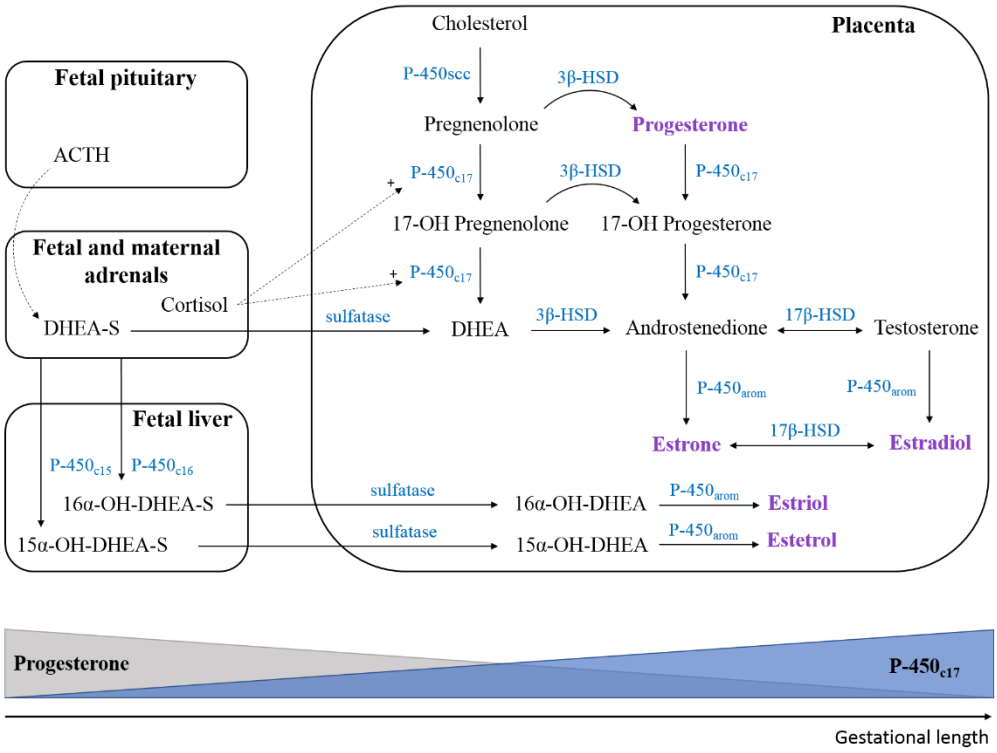


Figure 1. Steroid biosynthetic pathway for the conversion of substrate cholesterol to progesterone and estrogens during pregnancy. Enzymes are written in blue, main hormones for pregnancy are written in purple. Dashed arrows represent indirect pathways. Adapted from "Placental steroidogenesis in primate pregnancy", by G.J. Pepe and E.D. Albrecht, (pp889-898), 1998, Boston, Academic Press.

However, due to the lack of P-450$_{c17}$ activity in the placenta during the first half of gestation, estrogen biosynthesis requires the supply of dehydroepiandrosterone sulfate (DHEA-S) from fetal and maternal adrenals[3]. The sulfatase enzyme hydrolyzes DHEA-S into dehydroepiandrosterone (DHEA) which, in turn, is converted by the 3β-HSD into androstenedione and later into testosterone by the 17β-hydroxysteroid dehydrogenases (17β-HSD). These hormones are later converted by aromatase enzymes (P-450$_{arom}$) into estrone and estradiol. Throughout pregnancy, DHEA and DHEA-S levels remain constant, while estrogen production rates progressively increase.

The placenta also produces estriol whose level exceeds estrone and estradiol levels during late pregnancy. Contrary to these 2 estrogens, estriol production exclusively relies on fetal supplies, and thus reflects the steroidogenic activity of the fetal hypothalamic-pituitary-adrenal axis (HPA axis)[5]. In the fetal liver, the 16α-hydroxylase enzyme (P-450$_{c16}$) converts DHEA-S into 16α-hydroxydehydroepiandrosterone sulfate (16α-OH-DHEA-S) which, in turn, is converted to 16α-hydroxydehydroepiandrosterone (16α-OH-DHEA) in the placenta, and finally aromatized to estriol. In humans, around the 10-15th week of pregnancy, estriol levels rapidly rise in response to the increase in DHEA-S production by the fetal adrenals.

The last estrogen present in the maternal circulation during pregnancy is estetrol. Like estriol, estetrol production is solely relying on fetal precursors. In the fetal liver, the 15α-hydroxylase enzyme (P-450_{c15}) converts DHEA-S into 15α-hydroxydehydroepiandrosterone sulfate (15α-OH-DHEA-S) which, in turn, is converted into 15α-hydroxydehydroepiandrosterone (15α-OH-DHEA) in the placenta, and finally aromatized to estetrol. Estetrol levels continuously increase after midgestation in response to the increased steroidogenic activity of the fetal adrenals. Yet, as of now, little is known about its physiological role during pregnancy. Nonetheless, due to their distinct biosynthesis, estriol and estetrol echo the steroidogenic activity of the fetal adrenals, and thus reflect the growth of the fetal HPA axis during pregnancy.

In most subprimate mammals, parturition is preceded by a sharp rise in maternal estrogen levels[8,16]. In these species, estrogen production is low during most of pregnancy because of the lack of aromatizable C_{19} substrates (DHEA-S, 16α-OH-DHEA-S and 15α-OH-DHEA-S). At term, the rise in fetal adrenal cortisol levels induces the increase in estrogen levels by promoting the conversion of progesterone into androgens (see 1.1.1.3.)[17]. This shifts the maternal estrogen/progesterone ratio at term, leading to the changes in the uterus that are required for parturition.

Contrary to these species, in humans, estrogens levels progressively rise over the final weeks of gestation due to an increased DHEA-S production by the fetal adrenals[13,18]. However, there is no abrupt rise in estrogens levels right before parturition.

High levels of estrogens at term promote uterine contractility during active labor by (1) promoting the accumulation and storage of prostaglandin precursors[18], (2) stimulating prostaglandin release from the fetal membranes[18], (3) increasing myometrial responsiveness to uterotonic agents such as oxytocin (OT) through an estrogen receptor-mediated increase in the number of oxytocin receptors (OTR)[8,19], and (4) promoting gap junction formation in the myometrium[7,20].

1.1.1.3. Prostaglandins

Prostaglandins are lipid autacoids derived from arachidonic acid (AA) which stimulate uterine contractility at the time of parturition. During pregnancy, AA coming from the fetal membranes is synthetized into prostaglandins in the placenta (Figure 2).

AA liberation from the fetal membranes can be accomplished by both direct and indirect pathways. The direct pathway relies on the activation of the phospholipase A_2 enzyme (PLA_2)

which hydrolyzes phospholipids into AA. The indirect pathway depends on the activation of the phospholipase C enzyme (PLC). PLC hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG is later hydrolyzed by PLA_2 into AA.

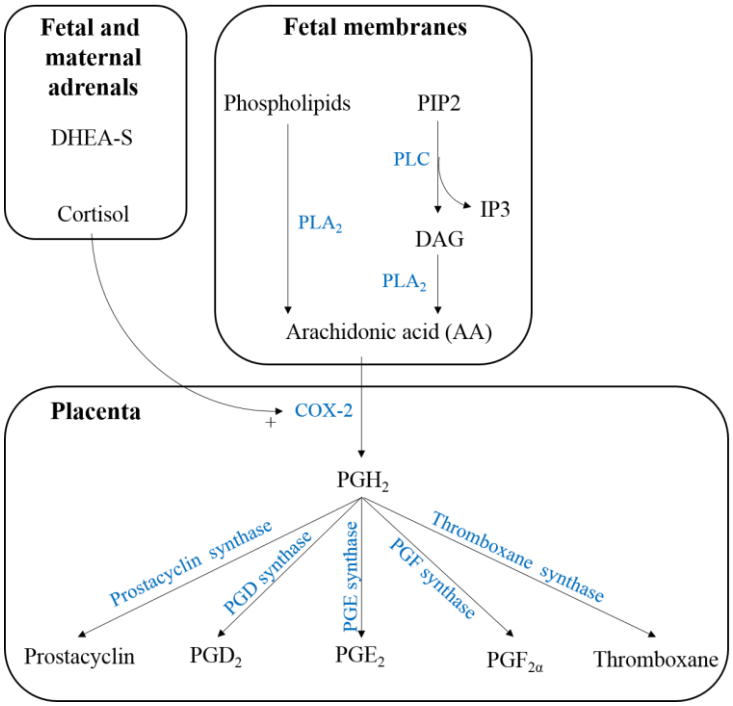


Figure 2. Prostaglandin synthesis pathway during pregnancy. Enzymes are written in blue. Adapted from "Cyclooxygenase Isozymes: The Biology of Prostaglandin Synthesis and Inhibition", by D.L. Simmons et al., 2004, *Pharmacological Reviews,* 56 (3): 387-437.

In the placenta, AA is oxidized by the cyclooxygenase-2 (COX-2) enzyme, resulting in the production of prostaglandin H_2 (PGH_2). The final step is the conversion of PGH_2 into one of the biologically active prostaglandins: prostacyclin by the prostacyclin synthase; prostaglandins D (PGD_2) by the prostaglandin D synthase, E (PGE_2) by the prostaglandin E synthase, and F ($PGF_{2\alpha}$) by the prostaglandin F synthase; and thromboxane by the thromboxane synthase.

The synthesis of prostaglandins is modulated by the availability of PLA_2 and AA. PLA_2, under the control of estrogen and progesterone, increases during late gestation[21,22]. Indeed, high progesterone and low estrogen levels stabilize the lysosomal membranes, while opposite hormonal levels lead to the release of PLA_2 from lysosomes[23].

AA levels begin to rise during mid-pregnancy in the maternal plasma while they increase right before the onset of labor in the amniotic fluid[24]. The increase in PLA_2 activity close to labor

also induces the release of stored AA from the chorioamnion into the decidua to provide substrate for prostaglandin synthesis[25].

1.1.1.4. Corticotropin releasing hormone (CRH)

CRH is a peptide hormone released by neurons located in the hypothalamus, that stimulates the secretion of pituitary adrenocorticotropic hormone (ACTH) and production of adrenal cortisol.

In pregnant women, from the second trimester onwards, placenta and fetal membranes become the major source of maternal CRH. This switch is controlled by cortisol which inhibits hypothalamic CRH and pituitary ACTH release, while stimulating CRH release by the placenta and fetal membranes[26,27]. Near term, the effects of CRH are strengthened by a fall in maternal plasma CRH-binding protein[28].

CRH activates the fetal HPA axis, resulting in enhanced fetal pituitary ACTH secretion and adrenal cortisol production. In addition, CRH also enhances the production of prostaglandins by the placenta and fetal membranes[29].

1.1.2. Fetal hypothalamo-pituitary-adrenal axis (HPA axis)

The fetal HPA axis is a major neuroendocrine system whose activation results in the synthesis and release of many hormones including cortisol, ACTH, and oxytocin. These hormones are at the center of mechanisms controlling parturition, with the fetal brain "fine-tuning" the timing of parturition while the fetal adrenal gland initiates and maintains labor. Indeed, monkey fetuses that underwent hypophysectomy have a delayed parturition[30]. Moreover, in human anencephalic fetuses with hypothalamic or pituitary atrophies, the timing of parturition is broadly distributed with premature and postmature births happening, whereas in the control group, birth occurs at term within two weeks of the mean[31].

1.1.2.1. Cortisol

Cortisol is a steroid hormone belonging to the glucocorticoid class and produced by the adrenal gland. During gestation, cortisol is the main hormone allowing organ maturation that is indispensable for neonatal adaptation at birth. In most species, cortisol levels in the fetal circulation rise during late pregnancy to reach peak levels during labor and a few hours after term delivery[32].

In sheep, this increase is linked to the maturation of the HPA axis during the last 2-3 weeks of gestation[32]. In humans, although the process is not well known, umbilical arterial cortisol levels

are found higher in spontaneous parturition than in fetuses delivered by cesarean section (C-section). The cortisol excess has been suggested to come from the fetal adrenal gland since most of the maternal cortisol is metabolized into inactive cortisone in the placenta[33].

At term, elevated fetal cortisol levels are proposed to stimulate COX-2 expression, causing an increase in the production of prostaglandins (Figure 2). Prostaglandins, in turn, stimulate P-450_{c17} expression, enhancing the conversion of progesterone into androgens[34] (Figure 1). Androgens are later metabolized into estrogens, whose increased synthesis in the placenta is required for the initiation of parturition, as described in 1.1.1.2.

1.1.2.2. Adrenocorticotropic hormone (ACTH)

ACTH is a polypeptide tropic hormone secreted by the anterior pituitary gland whose role during pregnancy is to increase the synthesis of estrogens.

ACTH secretion leads to the abundant release of DHEA-S from the fetal adrenal (Figure 1) in order to overcome the lack of DHEA in the placenta during most of gestation[35]. DHEA-S is used in the placenta to synthetize estrogens, as described in 1.2.1.2.

1.1.2.3. Oxytocin (OT)

OT is a neuropeptide hormone synthetized by magnocellular neurons located in the paraventricular, supraoptic and accessory nuclei of the hypothalamus, and secreted in a pulsatile manner by the neurohypophysis[36]. It is worth mentioning that OT is also synthetized by parvocellular neurons (located in the paraventricular, supraoptic and suprachiasmatic nuclei of the hypothalamus) projecting to the brainstem and spinal cord to act in a non-neuroendocrine way[37]. During late gestation, OT indirectly enhances uterine smooth muscle contractility by promoting prostaglandin synthesis[38].

In sheep and monkeys, there is a progressive increase in uterine contractility toward the end of gestation. This is related to an increase in maternal oxytocin levels during late pregnancy as infusion of a specific oxytocin antagonist attenuates these contractions[39–41].

In humans, maternal OT plasma levels do not increase before the onset of parturition even though OT levels in the amniotic fluid and umbilical blood are increased at term[42–44]. This may be due to the fetal OT secretion which progressively increase starting at the 14th week of gestation[45]. Fetal OT, as seen in the guinea pig, later crosses the fetal membranes[46] to interact with OTRs in the decidua, resulting in the activation of prostaglandin synthesis necessary during active labor [38]. This will be further described in 1.3.2.

1.1.2.4. Arginine-vasopressin (AVP)

AVP, a hormone synthetized by magnocellular and parvocellular neurons and secreted by the neurohypophysis, has received less attention for its role in parturition than its close analog hormone OT.

AVP is thought to participate during late labor and expulsion phases rather than initiating labor. Indeed, in sheep, plasma AVP levels are low during early phases of labor but rise during active labor and are associated with an increased myometrial sensitivity after labor[47,48]. Noteworthy, in humans, this rise is due to an increase in fetal rather than maternal HPA axis production, and the myometrial sensitivity to AVP was not shown to be associated with an increased AVP receptor type 1a expression[49]. In addition, during late gestation, AVP, through the AVP receptor type 1b, stimulates the fetal secretion of ACTH, while cortisol inhibits this peptide-induced release[50].

However, rather than participating in labor *per se*, AVP has been hypothesized to increase in response to the decrease in arterial pH and oxygen tension during labor's late phases[47]. Indeed, AVP secretion, independently of OT secretion, increases in response to stressful stimuli such as hypoxia and hemorrhage[51,52]. Moreover, in pregnant women going under C-section delivery, administration of AVP after removal of the placenta decreases blood loss[53]. Therefore, AVP secretion seems to rely on fetal HPA maturation during pregnancy and its levels increase in response to stressful events to counterbalance negative effects.

1.2. Active labor

The uterine myometrium, responsible for the coordination of muscular contractions, plays an important role in the mechanism of labor. It must transform from a quiescent state with dyssynchronous contractions, to a highly excitable state wherein contractions are synchronized and coordinated to promote cervix dilatation and fetus expulsion.

1.2.1. Uterine smooth muscle

The uterine smooth muscle is composed of myocytes which allow the tension or relaxation of the muscle. Three main types of contractile proteins are found in the sarcoplasm of these cells: actin, myosin and intermediate filaments (consisting largely of vimentin and desmin). Actin and myosin are organized in dense bodies. These bodies are attached to the intermediate filaments which are anchored to the adherens junctions of the sarcolemma (cell membrane).

When dense bodies contract, the force is transduced to the sarcolemma through intermediate filaments and adherens junctions[54,55].

Actin thin filaments are composed of an α-helical coil of actin filaments and associated proteins tropomyosin and caldesmon. Three isoforms are expressed in myometrial cells: α, β and ɣ types. While α-actin levels remain invariant throughout pregnancy, ɣ-actin isoform levels seem to increase and change localization towards term[56,57]. Myosin thick filaments consist of two heavy chains (MHC) forming a rod-like coiled-coil structure, two regulatory light chains (MLC_{20}) and two essential light chains (MLC_{17}). MHC are organized into three domains: the globular head domain, the α-helical neck region and the tail domain[54]. The head, containing actin- and adenosine triphosphate (ATP)-binding sites, converts the chemical energy of ATP hydrolysis into mechanical energy by interacting with actin to induce contractions. The neck region consists of MLC_{20} and MLC_{17} which regulate the interaction of the head domain with actin. The tail domain, by its binding sites, dictates the specificity of myosin within the cell[54].

The interaction between these two proteins is dependent on the intracellular calcium concentration ($[Ca^{2+}]_i$) which increases with the entry of Ca^{2+} from the extracellular fluid through voltage-gated L-type Ca^{2+} channels and the release of Ca^{2+} from intracellular stores in the sarcoplasmic reticulum[58]. The increase in $[Ca^{2+}]_i$ activates the Ca^{2+}-dependent protein calmodulin (CaM)[58]. CaM, in turn, activates the myosin light-chain kinase (MLCK), leading to the serine/threonine phosphorylation of MLC_{20}. Phosphorylation of MLC_{20} by MLCK activates the myosin head which binds to actin. The energy released from ATP by the head myosin ATPase activity results in subsequent cross-bridge cycling for muscle contraction[58] (Figure 3).

1.2.2. Hormonal control of the uterine myometrium

OT has a dual role in spontaneous labor: the activation of the calcium-mediated uterine contraction, and the increase of prostaglandins production[59].

In mammals, elevated plasma levels of OT are observed during labor[60,61]. In many species, this is associated to a pulsatile secretion of OT which is maximal during fetal expulsion[41,62]. However, this remains controversial for humans since variations in OT concentrations have been reported during human pregnancy and labor[62–65].

At term, the number of OTR drastically increases in the myometrium and leads to an increased sensitivity to OT[59]. Binding of OT to OTR leads to the activation of the $G\alpha_{q/11}$ protein which, in turn, activates PLC. PLC then hydrolyzes PIP2 into IP3 and DAG (Figure 3).

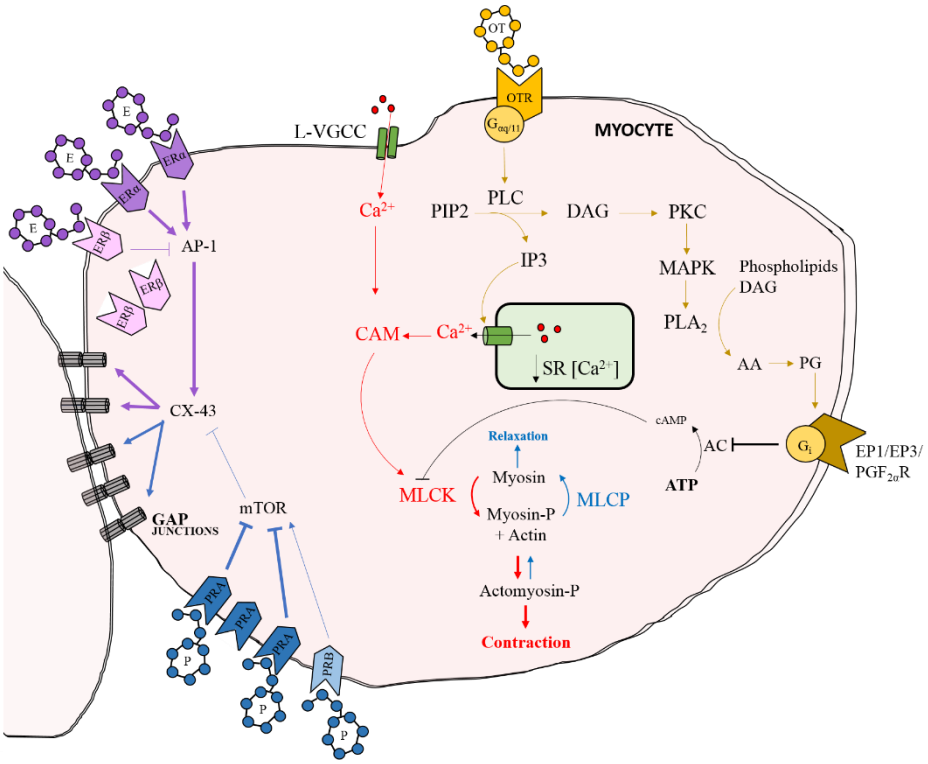


Figure 3. Regulation of the hormonal signaling in the myometrium to induce synchronous contractions during active labor. E: estrogens and P: progesterone. → activation, ⊣ inhibition. Adapted from "Oxytocin: its mechanism of action and receptor signaling in the myometrium" by S. Arrowsmith et al., 2014, *Journal of Neuroendocrinology*, 26(6): 356-69.

Binding of IP3 to its receptor results in the release of Ca^{2+} ions from the intracellular stores to the sarcoplasmic reticulum[66]. This increase in $[Ca^{2+}]_i$ initiates smooth muscle contraction as described in 1.2.1. The binding of OT to OTR also activates L-type calcium channels in response to membrane depolarization[67] and to the entrance of extracellular Ca^{2+} in the sarcoplasmic reticulum, further promoting smooth muscle contraction.

In addition, DAG activates protein kinase C (PKC) which activates other intracellular targets including the mitogen-activated protein kinase (MAPK) and the Rho kinase pathways (Figure 3). The Rho kinase pathway is associated with cell migration, cell cycle control and cell contractility, while the MAPK pathway results in an increase in cytosolic phospholipase A_2 ($cPLA_2$) activity. The activity of $cPLA_2$ is increased during labor at term in human placenta, fetal membranes and decidua, resulting in an increase in prostaglandins biosynthesis[68]. This finding is further supported by the modification of AA levels during parturition. Following labor, AA levels are lower in the chorioamnion than before the onset of parturition, suggesting a release of the acid from glycerophospholipids before and during labor. Moreover, the ratio

AA/free fatty acids in the plasma decreases, implying that the free AA are rapidly metabolized to prostaglandins[24].

Throughout gestation, PGE2 and $PGF_{2\alpha}$ levels increase in a time-dependent manner in the maternal human plasma, amniotic fluid and urine with a stronger rise at term prior to the onset of labor and plateauing during parturition[69]. Their action is modulated through prostaglandins receptors which are sorted by their effects on smooth muscle: relaxation or contraction. Receptors associated with relaxation are PGE2 receptor subtypes 2 and 4, and PGD2 receptor; while receptors associated with contraction are PGE2 receptor subtypes 1 (EP1) and 3 (EP3), and $PGF_{2\alpha}$ receptor (Figure 3).

Prostaglandins receptors are G-protein-coupled receptors whose response is mediated by their coupling to either Gs or Gi proteins. Gs activation stimulates adenylyl cyclase which hydrolyzes ATP in cAMP. cAMP increased levels activate protein kinase A which in turn inhibits MLCK therefore leading to muscle relaxation. On the other hand, activation of Gi protein inhibits adenylyl cyclase (AC) resulting in decreased levels of cAMP and subsequently smooth muscle contraction[70]. Receptors expression levels vary through pregnancy, with an increase in EP1 and down-regulation of EP3 in the lower segment of the myometrium of women in labor at term, suggesting that EP1 is more likely to mediate the contractile effects during labor[71]. In contrast, EP2 mRNA levels are higher in preterm non-laboring women compared to women at term[72]. Therefore, this up-regulation of contractile receptors and loss of relaxation receptors contribute to the switch from a quiescent myometrial state to a highly excitable state.

1.2.3. Synchronization of muscle contraction

Labor is associated with a shift from dyssynchronous to synchronized contractions resulting in the dilatation of the cervix and later on the expulsion of the fetus. This coordination is under the control of gap junctions which enhance electrical conductivity by permitting the direct exchange of ions, second messengers and small metabolites between cells. Gap junctions consist of two hemichannels, one on each plasma membrane of adjacent cells, resulting from the oligomerization of six connexins. In the myometrium, the dominant connexin in gap junctions is connexin 43 (CX-43)[73]. The number of gap junctions present in the myometrium rises during pregnancy and is maximum during labor[74]. This increase is positively correlated with increased cervical dilation or uterine contractions frequency. In fact, the importance of gap junctions has been demonstrated in pregnant women giving birth naturally and by C-section deliver, since myometrial tissues from women giving birth by C-section delivery either at term

or preterm, the number of gap junctions was lower than in women in labor at term or premature labor[75].

CX-43 synthesis and the initiation of gap junction formation is hormonally regulated by progesterone, estrogens and prostaglandins[20]. The ratio estrogens/progesterone is low during gestation, but it increases at term and is associated with a rise in CX-43 expression at this time[74,76]. Typically, binding of estrogens to its receptor (ER) activates CX-43 expression while progesterone binding to its receptor (PR) inhibits CX-43 synthesis by trans-repressing CX-43 transcription[76]. However, this classical view has been adjusted in recent years. Estrogens can bind to two receptors subtypes, ERα and ERβ, which have opposite regulatory actions by activating and inhibiting respectively AP-1 transcription factor activity whose stimulation induces CX-43 gene transcription[77]. In human myometrial cells, ERα is the predominant form when non-pregnant, whereas ERβ is the predominant form in pregnant women's myometrium[77]. During labor, ERβ shifts from a fast mobility isoform to a slow mobility isoform and is associated with increased CX-43 expression and decreased ERβ levels. In fact, the down-regulation of ERβ levels and function might lift the AP-1 inhibition therefore leading to the activation of CX-43 gene expression (Figure 3). Regarding progesterone, its actions might be modulated differently through its binding to either PRA or PRB[78]. During pregnancy, binding of progesterone to its receptor PRB activates the mTOR pathway which, in turn, represses the expression of CX-43. On the other hand, PRA expression in the nucleus increases close to the onset of labor and promotes gap junction formation via the inhibition of the mTOR pathway[78] (Figure 3). Therefore, estrogens and progesterone play important roles in regulating myometrial gene expression and protein synthesis, notably for CX-43, during pregnancy and labor. Finally, prostaglandins (thromboxane, $PGF_{2\alpha}$ and PGE_2) may stimulate gap junction development by controlling the connexins aggregation or by altering the fluidity of the membrane to promote the insertion of connexins into it[79].

Altogether, these physiological phenomena promote the rupture of fetal membranes, cervix dilation and lead to the expulsion of the fetus into its new environment.

1.3. Transition from intrauterine to extrauterine life

The transition from intrauterine to extrauterine life is a complex physiologic adaptation that involves essential mechanisms for the survival of the newborn, including endocrine, metabolic, respiratory, gastrointestinal, cardiovascular and neurological adaptations in a very short period of time.

1.3.1. Endocrine adaptations

The fetal hypothalamo-pituitary-adrenal axis (HPA axis) is a major neuroendocrine system whose activation results in the synthesis and release of many hormones including cortisol, catecholamines and thyroid hormones. These hormones are at the center of mechanisms controlling fetal readiness for birth, survival after birth and, in many species, initiation of parturition.

1.3.1.1. Cortisol

HPA-derived cortisol is the principal regulatory hormone that allows organ maturation for the adaptation of the fetus at birth[80]. The increase in fetal cortisol levels is associated with multiple physiological changes such as enzymes synthesis to facilitate the neonatal adaptation.

During pregnancy, the fetus is in a constant thermoneutral environment which does not require for the thyroid to modulate its hormonal secretion in response to acute changes in metabolic rate. At birth, the thyroid, under the control of cortisol, switches from a moderate to a highly activated state to meet the neonatal requirements. This will be further described in 1.3.1.3.

In fetal lungs, the rise in cortisol levels stimulates surfactant synthesis and secretion, connective tissue maturation, alveolar epithelial differentiation, lung liquid resorption and glycogenolysis. This will be further described in 1.3.3.

The cortisol surge also promotes energy metabolism in the liver. During pregnancy, glucose from the mother is constantly received by the fetus through transplacental passage. This source of energy is abruptly stopped at birth and the fetus must rapidly adapt. In this context, the liver becomes an alternative source of energy by providing glucose from glycogen stores and promoting gluconeogenesis. This will be further described in 1.3.2.1.

1.3.1.2. Catecholamines

Catecholamines are monoamine compounds which comprises norepinephrine, epinephrine and dopamine. These compounds are released by the fetus' HPA axis in response to stress. At term, the fetus experiences a catecholamines surge with fetal plasma catecholamines concentrations singularly higher than in resting adults[81]. Moreover, the amniotic norepinephrine, epinephrine, and particularly dopamine concentrations are also increased toward term[82]. The catecholamines rise is a response to the stress of being born. Indeed, in babies who suffered from intrauterine asphyxia (e.g. after breech deliveries), catecholamines levels are even higher than in term infants delivered uneventfully[81]. This surge drives vital adaptive responses to birth such as the

fetal circulation during the hypoxic period while going through the birth canal, increase in blood pressure following birth, adaptation of the energy metabolism, and initiation of thermogenesis in addition to stimulating prostaglandins synthesis[83].

1.3.1.3. Thyroid hormones

During late gestation, the thyroid axis matures in parallel to the increase in cortisol levels. This results in (1) increased levels of thyroid stimulating hormones (TSH), tri-iodothyronine (T_3) and thyroxine (T_4) through pregnancy, and (2) decreased levels of reverse T_3 (rT_3), an isomer of T_3. At birth, the cortisol surge is associated to a quick rise and decrease in TSH levels, further increase in T_3 and T_4 levels, and decrease in rT_3 levels[84]. T_3 and T_4 high supplies after birth are required for the thyroid to maintain fetal body temperature and cope with breathing. However, thyroid hormones might have a more important effect before birth than after. Inhibition of thyroid function prior to birth altered postnatal adaptation and thermogenesis in newborn lambs while acute ablation of thyroid function at birth had a moderate effect on these parameters[85]. Therefore, even though thyroid hormones act as modulator of endocrine adaptation after birth, their role before birth is as important to support and prepare the fetus to external life.

1.3.2. Metabolic adaptations

1.3.2.1. Energy metabolism

In late pregnancy, the fetus starts storing glucose and other substrates in the form of glycogen and fat required at birth. In sheep, this process is positively correlated to the cortisol surge observed prenatally and results from the activation of glycogen synthetase[86]. However, in the liver, the gluconeogenesis pathway (resulting in the generation of glucose from non-carbohydrate carbon substrates) only activates shortly before birth in sheep and may only be activated at birth in humans[87,88]. Indeed, gluconeogenesis is dependent on the appearance of its rate-limiting enzyme, the phosphoenolpyruvate carboxykinase (PEPCK), which is induced by the plasmatic rise in glucagon and fall in insulin occurring immediately after birth[88]. PEPCK activation leads to increased glucose plasma levels to counteract the rapid decrease resulting from the abrupt stopping of maternal supply. In addition, the catecholamines surge at birth enhances the (1) rates of aerobic glycolysis, resulting in ATP production, (2) glucose release from gluconeogenesis, and (3) inhibition of insulin-mediated glycogenesis[89]. The adaptation of the fetus' metabolism at birth promotes the newborn supplies change from a high-carbohydrate and low-fat diet to a high-fat and low-carbohydrate diet.

1.3.2.2. Thermoregulation

During late gestation, the fetus develops its thermogenic response potential by increasing its brown adipose tissue reserves around the kidney and in the intrascapular areas of the back[90]. At birth, fetal body temperature suddenly drops due to the evaporation of the amniotic fluid from the skin and the exposition to cool surrounding air. In response to these cold stimuli to the skin as well as increased oxygenation, the sympathetic nervous system releases norepinephrine which, in turn, activates the mitochondrial membrane protein uncoupling protein 1 (UCP1)[83]. In the mitochondria of brown adipose tissue, UCP1 uncouples oxidative metabolism from ATP synthesis, resulting in the release of heat. UCP1 synthesis increases during late gestation in response to the increase in fetal cortisol levels and the local conversion of T_4 to T_3[91]. In addition to thermoregulation controlled by UCP1, term infants can also generate heat by shivering thermogenesis[92]. This mechanism of second importance at birth relies on the non-purposeful increase in the metabolism of skeletal muscle in response to variations in thermal conditions.

1.3.3. Respiratory adaptations

1.3.3.1. Lung fluids and maturation

Prior to birth, gas exchanges occur across the placenta as the fetal lung airways are immature and liquid-filled. The fetal lung fluid is constituted of a low protein content, and a high chloride content due to the active transport of chloride from the lung interstitial fluid to the airway epithelium[93]. Its production and maintenance in a normal volume is essential for normal lung organogenesis which is required for the switch from placental to pulmonary exchanges at birth.

Lung maturation is an essential step for the fetal lung to support gas exchanges and breathing. During the late gestational to postnatal periods, about 4 million distal saccules form on the terminal bronchioles which represent the last subdivision of the passages that supply air[94]. In addition, the alveolar epithelial cells differentiate into mature type I pneumocytes and type II pneumocytes which coat each saccule. The type II pneumocytes bear lamellar bodies that store and secrete the essential biophysically active components of the surfactant. The number of lamellar bodies containing surfactant and released into the fetal lung fluid is positively correlated with lung maturation[83]. In humans, starting in the last few weeks of pregnancy until 6 months postnatal, fetal lung maturation terminates with the alveolar phase where alveoli form from saccules to expand the gas exchange surface that will be recruited for breathing[95].

1.3.3.2. Breathing at birth

In rats, fetal lung fluid production and volume decrease before the onset of birth and furthermore with labor[96]. This fluid clearance is vital and dependent on the endocrine adaptations involved in the passage from intrauterine to extrauterine life. Indeed, at term, the increase in cortisol, catecholamines and thyroid hormones (mostly T_3) levels induces the stopping of the chloride-mediated secretion of lung fluid and the activation of the absorbing Na^+/K^+-ATPase pumps of type II pneumocytes[83]. The activation of these pumps leads to the passage of sodium from these cells to the interstitium along with the passive transfer of water, thus leading to the removing of fluid from the airways. In parallel, the surfactant secretion increases under the influence of external factors. Purinergic agents such as ATP promote pre-delivery secretion while the catecholamines surge, via β-receptors stimulation, induces an increase in surfactant secretion during labor. In rats, low catecholamines levels have been correlated with respiratory distress or transient tachypnea[97].

At birth, the initiation of ventilation causes an alveolar stretching and leads to the deformation of type II pneumocytes, sending a signal to trigger surfactant secretion. The catecholamines surge further stimulates this process, resulting in a fetal lung containing 5 to 20-fold higher surfactant amount than the adult lung[83]. These high concentrations ensure the term fetus to have an adequate surfactant amount for the transition to air breathing. Then, the clamping of the umbilical cord is determinant for setting up continuous breathing. Indeed, fetal sheep exposed at birth to a placentally derived substance such as prostaglandins stop breathing by themselves suggesting that they may try to rely on placental breathing[98]. Furthermore, breathing does not begin in the presence of severe hypoxia, showing that initiation of breathing also necessitates changes in the partial pressure of carbon dioxide (PCO_2) and oxygen (PO_2) levels in the blood[83]. Therefore, in term infants, air breathing is ensured by both prenatal and postnatal factors which favor normal breathing at birth.

1.3.4. Cardiovascular adaptations

Cardiovascular reorganization must take place within the first minutes of life in response to the removal of the low resistance placenta as the source of fetal gas exchange and nutrition. This critical process depends on the appropriate timing of cord clamping and lung aeration which, out of sequence, may expose the newborn to cardiovascular instability and complications.

In the fetus, the oxygenated blood comes from the placenta via the umbilical cord and ductus venous. The ductus venous blood enters the right atrium from the inferior vena cava, goes to

the left atrium by the foramen ovule and is consequently delivered to the brain and coronary circulation. The predominant ventricle is the right one which redirects the right ventricular output (RVO) through the ductus arteriosus and into the systemic circulation. This is due to the low oxygen tension and pulmonary blood flow (PBF) which suppress the synthesis and release of nitric oxide (NO) and prostaglandin I_2 (PGI_2) from the pulmonary endothelium, contributing to high pulmonary vascular resistance (PVR)[99].

At birth, the umbilical cord clamping reduces the umbilical venous return to the heart which was the source of preload for the left ventricle before birth, and directly increases systemic vascular resistance[100]. These changes induce a rapid increase in arterial pressure which is correlated with an increase in cerebral blood flow. Then, the cardiac output decreases, causing cerebral blood flow to decrease and arterial blood pressure to stabilize. The cardiac output remains low until ventilation starts and PBF increases, restoring the venous return and left ventricular preload, and leading to the recovery of the cardiac output[101]. This regulation of the cardiac output is required for basal metabolism, breathing and thermogenesis activities. In addition, the increase in cardiac output at last is correlated with a rise in oxygen consumption and blood flow in the lungs, heart, kidney and gastrointestinal tract.

In addition, the removal of the low resistance placenta concomitant with lung ventilation and oxygenation induces an increase in NO and PGI_2 levels leading to the dilatation of pulmonary vessels and the decrease of PVR[83]. PVR decrease and PBF increase are associated with the closure of the ductus arteriosus[102]. Therefore, the cardiac reorganization at birth leads to PBF becoming the sole receiver of RVO and the sole source of preload for the left ventricle[103]. These transitions at birth are modulated by hormones. Cortisol, catecholamines and thyroid hormones induce an increase in β-receptors signaling, increasing cAMP and leading to the increase in blood pressure, cardiac output and left ventricular contractibility[104]. Altogether, this reorganization causes the closing of major vascular shunts, leading to the anatomical separation of the pulmonary and systemic circulations as well as the separation of the heart into left and right sides.

1.3.5. Gastrointestinal adaptations

Microbial colonization of the gut at birth is fundamental for the later development of the gastrointestinal tract, metabolic, and immune systems[105]. Moreover, the microbiota communicates with the brain, and gut dysbiosis has been shown to alter brain synaptogenesis,

regulation of neurotransmitters and neurotrophic factors, regulation of the development and maturation of microglia, and behavior in mice[106–108].

In pregnant women, the composition of two main body sites (vaginal and intestine) responsible for microbial transmission to the newborn during vaginal delivery changes throughout gestation. Indeed, bacterial diversity decreases as women progress through pregnancy[109]. The vaginal microbiome is mainly composed of *Lactobacilli*, *Clostridiales*, *Bacteroidales*, and *Actinomycetales*, with a higher prevalence of specific species such as *Lactobacilli* during gestational late ages[110]. The increasing proportion of *Lactobacilli* promotes the maintenance of a low pH to limit bacterial diversity and prevent bacteria from ascending to the uterus and further infecting the amniotic fluid, placenta and fetus[111]. The gut microbiome also changes with an increase in proinflammatory Proteobacteria, and a decrease in anti-inflammatory *Faecalibacterium prausnitzii* from the first to the third semester[109].

At birth, the newborn is exposed to vaginal, fecal, and skin microbiota that results in massive bacterial colonization[112]. In addition, maternal breast milk contains bacteria such as *Bifidobacterium*, *Lactobacillus* and *Streptococcus* among others[113], thus breastfeeding promotes the colonization and maturation of the infant gut microbiome. The early microbiome further develops in response to food diet with organisms facilitating lactate utilization at first to anaerobic organisms necessary for the utilization of solid foods later[114].

1.3.6. Neurological adaptations

In addition to the previously described physiological adaptations, the fetus also undergoes brain adjustments at birth. These changes are crucial for the fetal brain to continue to develop after the stressful and traumatic event that is birth. Yet, very little is known about it.

1.3.6.1. Brain growth

The human brain progressively grows during the fetal period and undergoes an impressive transformation during the perinatal period through the first two years of life[115]. During late gestation, the brain changes from a non-complex bi-lobed organ into one resembling the adult brain and presenting sulci and gyri. This transformation reflects a rapid growth and development of the brain during the perinatal period. Interestingly, this growth is dependent on the weight of the baby at the time of birth[116]. In term babies with an appropriate weight, brain growth slows down right before birth while a spurt is observed right after. However, this birth

slowdown has not been seen for term infants with a small weight for their age nor for preterm infants.

These growth changes are correlated to nutrition which is known to influence early brain development. Indeed, nutrients that support energy, carbohydrate, protein, and fat metabolism affect the structural development of the brain through their signaling pathways[117]. Furthermore, the impact of nutrition on brain growth is greater during the perinatal period because of the high metabolic demands of the brain at that age[117]. Malnutrition at this age may mostly lead to intrauterine growth restriction in fetuses, inducing slower head growth, IQ reduction and increased risk of developing schizophrenia in adulthood[118,119].

1.3.6.2. Neuroprotection of the brain

In humans, vaginal delivery triggers the activation of an analgesic effect at birth which is seen by the dampened behavioral response of babies to both pain and cold stimuli[120]. This antinociceptive mechanism, necessary for the proper development of the brain and its protection during delivery, is mediated by both catecholamines and OT release.

At birth, high levels of catecholamines, and notably norepinephrine, are released following sympathoadrenal activation[121]. In the brain, norepinephrine may activate the α_2-adrenergic receptor and induce strong analgesic effects such as the ones seen with clonidine, an α_2-adrenergic agonist[122]. Dysfunction in catecholamine neurotransmission is involved in the onset of numerous neuropsychiatric disorders such as depression[123] and attention deficit disorder with hyperactivity[124].

In parallel, OT, whose levels increase during delivery, also exerts a protective action in the fetus at birth. In rats, the blockade of OTRs enhances pain sensitivity at birth while OT administration decreases it. These actions are mediated by modifications of intracellular chloride concentration ($[Cl^-]_i$) in the nociceptive neurons. Indeed, the depolarizing action of GABA (ɣ-aminobutyric acid; will be described later in 4.3.2.2.1.) and GABA-evoked calcium responses are reduced in these cells in rats administered with either OT or bumetanide (a NKCC1 chloride co-transporter antagonist)[125]. Moreover, during delivery, in hippocampal neurons, OT triggers a transient shift from a depolarizing to a hyperpolarizing action of GABA[126]. This oxytocin-mediated switch is crucial for brain development as its abolishment induces long-term behavioral consequences[127]. Yet, this finding has been recently challenged as one study showed that GABA is depolarizing throughout the whole perinatal period[128].

1.4. Premature delivery

Premature birth, defined in humans as giving birth before 37 weeks of gestation, is a serious cause of infant morbidity and mortality due to the complications associated to this type of birth[129]. In premature babies (preemies), the transition from intra- to extrauterine life is not physiologic because the pre-delivery adaptive processes are not yet fully developed when the baby is born.

1.4.1. Risk factors

Numerous factors have been associated with preterm birth and can be divided into two different categories: pregnancy-related and environmental-related problems.

Pregnancy-related problems include (1) cervical incompetence where the cervix is weak and begins to dilate before the onset of parturition[130], (2) birth defects of the uterus[131], (3) preeclampsia consisting of hypertension and proteinuria[132], (4) placenta previa involving a premature rupture of the membranes[133], and (5) having a previous premature birth[134].

Environment-related problems include (1) poor nutrition[135], (2) tobacco use[136], (3) chronic conditions such as diabetes[137], (4) infections[138,139], (5) maternal age[140], (6) body mass index of the mother[141,142], (7) drug use and physical trauma[143], (8) lack of prenatal care[144], and (9) low socioeconomic status[145]. Even though various elements have been found over years, a common cause for premature birth has yet to be identified.

1.4.2. Complications at birth

During the first weeks after premature birth, complications due to this non-physiological process are observed.

1.4.2.1. Metabolic adaptations

In preterm infants, the blood glucose decline at birth is greater than in term infants[146]. This drop is correlated to cortisol and catecholamines responses which are dysregulated at birth, with less cortisol and more catecholamines release. Low cortisol levels decrease the conversion of alanine into glucose and glycogen necessary for the metabolism at birth[147]. Furthermore, high gluconeogenic substrates levels are found in preemies while the activity of the microsomal glucose-6-phosphatase, the final enzyme of gluconeogenesis and glycogenolysis, is low compared to term infants[148]. Glycogen levels are also low, and preemies do not mobilize

adequately fat stores[149]. These results indicate that preemies have an inability to increase their metabolic processes at birth in response to the glucose drop.

1.4.2.2. Breathing adaptations

Numerous preemies do not have suitable spontaneous respirations at birth. Even though breathing patterns of term and preterm infants right after birth are similar, preemies have a higher number of expiratory hold pattern[150]. This might be an accommodation act in response to the difficulty for these infants to keep their lungs open and aerate them. Indeed, because of the immaturity of the lungs, surfactant secretion is inadequate to decrease surface tension and maintain functional residual capacity at birth. As a result, high surface forces are generated in the air interface across epithelial cells, distorting these cells and injuring the epithelium of small airways. This feature is distinguishable in preemies who died from respiratory distress syndrome[151]. In addition, in sheep, prematurity has been associated with increased pro-inflammatory cytokines mRNA expression (IL-1β, MCP-1, IL-6) in tracheal airways and positively correlated with an increased risk of bronchopulmonary dysplasia[152]. Altogether, at birth, the preterm lung is more prone to suffer from airway injuries during the initiation of breathing which may lead to persistent lung diseases.

1.4.2.3. Cardiovascular adaptations

In preterm babies, cardiovascular immaturity and hemodynamic instability are associated with glucocorticoids deficiency at birth[153]. Indeed, preterm birth happens before the occurrence of the physiological surge in cortisol (glucocorticoids). Complications resulting from this deficiency include the persistence of the ductus arteriosus and delays in PVR decrease and cardiac output increase[154]. Exogenous glucocorticoids treatment rectifies these complications, supporting the crucial role of glucocorticoids secretions in achieving normal circulatory function[155,156].

1.4.3. Brain complications

Improvement in neonatal care has increased the survival rate of preemies, but premature birth renders the infant more vulnerable to long-term adverse neurodevelopmental outcomes. To understand the impact of prematurity on the developing brain, neuroimaging techniques allow us to study volumetric and vasculature alterations in a non-invasive way.

1.4.3.1. Cerebral volumetric alterations

In children born prematurely, brain regions are differentially vulnerable during development and correlated with diverse neuropsychiatric impairments. In patients born premature and presenting neurological abnormalities, a disruption of corticothalamic connectivity is observed with volume reductions in the caudate nucleus[157], sensorimotor and mid-temporal cortex[158], hippocampus[159], secondary sulci[160], and sex-associated effects on grey matter and white matter[161]. However, these results must be taken with caution since at equivalent age to term, preterm infants with no neurological abnormalities do not seem to have smaller cerebral volumes than term infants[162].

1.4.3.2. Cerebral vasculature alterations

In babies born preterm, the cerebral vasculature is still developing, and the preemie is unable to regulate its cerebral blood flow[163]. This loss of autoregulation, also called pressure passive cerebral circulation, implies that when blood pressure falls at birth, so does the cerebral blood flow. This results in ischemia within the arterial end zones and white matter border zones. Infants with pressure passive cerebral circulation are more prone to develop germinal matrix-intraventricular hemorrhage and periventricular leukomalacia[163]. In addition, ischemia results in white matter injury by inducing pre-oligodendrocytes injury which leads to the activation of microglia and a decreased presence of neurons in the ventricular zone, subventricular zone and cerebral white matter[164].

Conclusion

Several physiological changes take place during parturition and early postnatal life, with alterations being present in premature birth. Summaries of the hormonal and organ changes are presented in Figure 4 and Table 1.

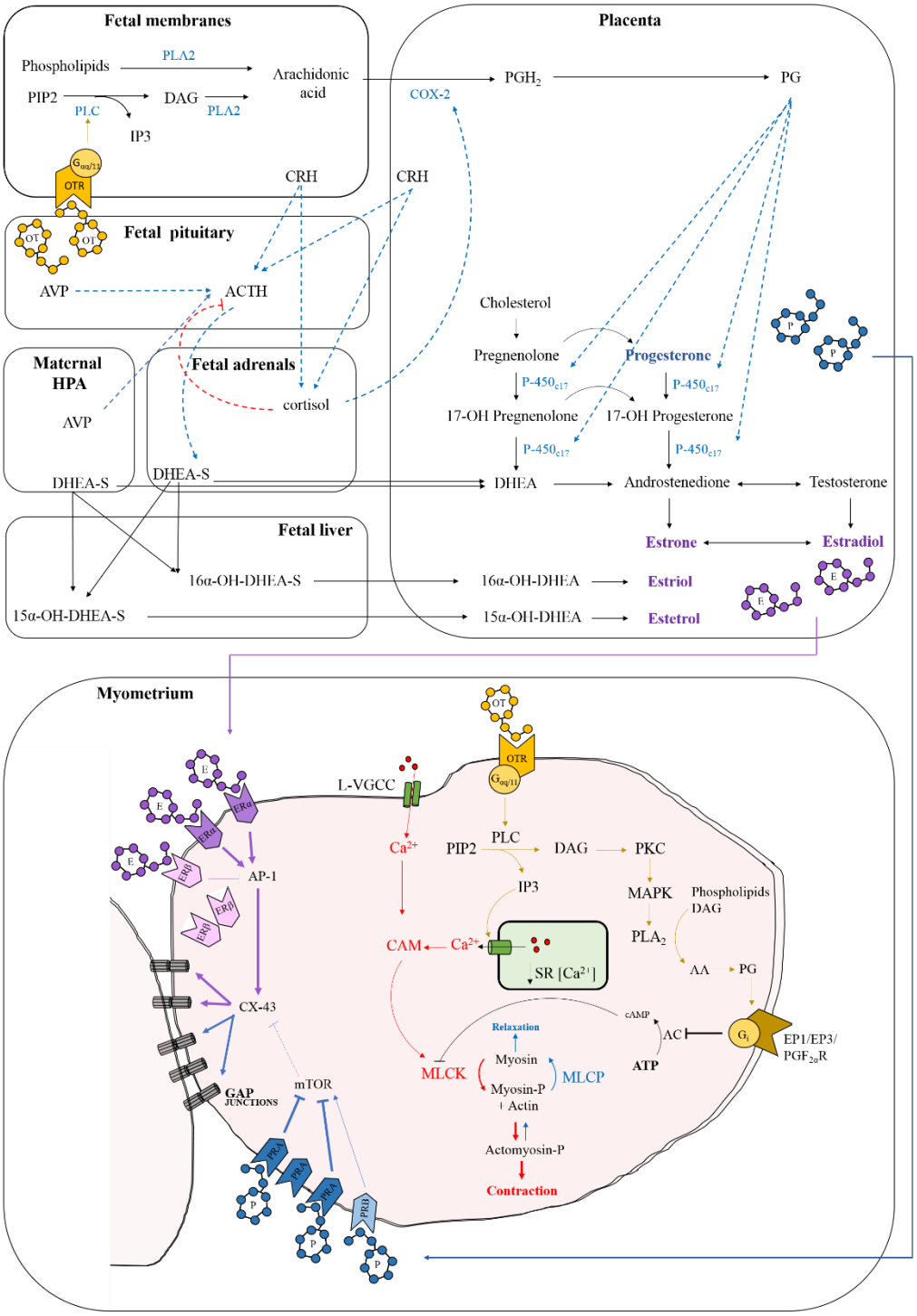


Figure 4. Summary of the physiological changes taking place during labor. Adapted from (1) "Placental steroidogenesis in primate pregnancy", by G.J. Pepe and E.D. Albrecht, (pp889-898), 1998, Boston, Academic Press. (2) "Cyclooxygenase Isozymes: The Biology of Prostaglandin Synthesis and Inhibition", by D.L. Simmons et al., 2004, *Pharmacological Reviews*, 56 (3): 387-437. (3) "Oxytocin: its mechanism of action and receptor signaling in the myometrium" by S. Arrowsmith et al., 2014, *Journal of Neuroendocrinology*, 26(6): 356-69.

Term delivery			
	Initiation of parturition	Active labor	Transition from intra- to extra-uterine life
Key organs	Placenta Fetal membranes Fetal HPA axis	Uterine smooth muscle	Endocrine adaptations Metabolic adaptations Breathing adaptations Cardiovascular adaptations Gastrointestinal adaptations Neurological adaptations
Key hormones and players	Progesterone Estrogens Prostaglandins CRH Cortisol ACTH OT	$[Ca^{2+}]_i$ Actin Myosin Intermediate filaments MLCK CaM OT OTR Prostaglandins Estrogens receptors Progesterone receptors CX-43	Cortisol Catecholamines Thyroid hormones Gluconeogenesis (PEPCK) Brown adipose tissue (UCP1) Surfactant Pulmonary vascular resistance (PVR) Pulmonary blood flow (PBF) Microbiota Brain growth OT GABA and $[Cl^-]_i$
Premature delivery			
Changes in key players	↓ cortisol ↑ catecholamines Altered gluconeogenesis Altered fat stores ↓ surfactant ↓ glucocorticoids Delay in PVR decrease Brain volume alterations Altered cerebral blood flow that could lead to ischemia		

Table 1. Summary of organs and molecules involved in parturition and their alteration during preterm delivery.

2. CESAREAN SECTION DELIVERY

Cesarean section, also known as C-section, is a surgical procedure used as an alternative to vaginal parturition when required by medical indication. However, the rise in C-section rates over the past decades has become a major public health concern and has caused worldwide debates due to the potential maternal and fetal perinatal risks, cost issues and inequity in access.

2.1. Etymology and history

Cesarean section has been a part of human culture since ancient times. Yet, the exact timeline and origin of the word remain debatable as of today. Popular legends attribute the origin of this term to the birth of Julius Caesar that believe the Roman Emperor was cut from his mother's womb[165]. However, scholars are more prone to believe in different origins. Some believe the phrase comes from the Latin verb "caedere" (in past participle "caesus") which mean "to cut". Others believe the name comes from the Roman decree "Lex Caesaria" which was established before Caesar's birth at the time of Numa Pompilius in 715-673 B.C. This law required the baby to be removed from the mother who was dying or died during parturition. Rather than for saving the baby, this law was first made to comply with Roman ritual and religious edicts which forbade the burial of a pregnant woman[165,166].

The operation was later conducted in hope to save the baby's life but never intended to preserve the mother's life. The first written record of a woman and its baby surviving a C-section comes from Switzerland. In 1500, Jacob Nufer, a sow gelder, performed the operation on his wife which had failed to give birth after few days of labor. One of the reasons of this success might be the presence of improved hygienic conditions in rural areas compared to hospitals at that time[166,167].

Over the past 200 years, progress in anesthesia, antiseptics and antibiotics have improved the survival of both mothers and babies and led to C-section delivery as we know of it today.

2.2. Epidemiology

Over the past decades, the prevalence of C-section delivery has dramatically increased[168]. It is now estimated to be 19.1% worldwide, with widespread disparities across developing and developed regions (Table 2).

Region/subregion[b] (coverage, %)	Change in rate (earliest and latest rates, %)	Absolute increase (%)	AARI[c] (% per year)
Africa (81.8)	**2.9–7.4**	**4.5**	**4.0**
Eastern Africa (96.3)	2.3–3.9	1.6	2.2
Middle Africa[d]	-	-	-
Northern Africa (97.3)	4.5–27.8	23.3	7.9
Southern Africa[d]	-	-	-
Western Africa	2.6–3.1	0.5	0.7
Asia (93.1)	**4.4–19.5**	**15.1**	**6.4**
Eastern Asia (97.8)	4.9–35.2	30.3	8.5
South-central Asia (96.4)	4–11.4	7.4	4.4
South-eastern Asia (84.0)	4.1–15	10.9	5.5
Western Asia (68.9)	6.3–28.1	21.8	6.4
Europe (98.1)	**11.2–25.0**	**13.8**	**3.4**
Eastern Europe (100)	7.8–23.7	15.9	4.7
Northern Europe (100)	11.1–22.4	11.3	3
Southern Europe (90.3)	16.3–31.1	14.8	2.7
Western Europe (100)	14.8–24.5	9.7	2.1
Latin America and the Caribbean (84.3)	**22.8–42.2**	**19.4**	**2.6**
Caribbean (67.5)	9.9–28.5	18.6	4.5
Central America (97.9)	14.8–38.4	23.6	4.1
Southern America (79.4)	28.4–45.8	17.4	2
Northern America (100)	**22.3–32.3**	**10**	**1.6**
Oceania (56.6)	**18.5–32.6**	**14.1**	**2.4**
Australia/New Zealand (100)	18.5–32.6	14.1	2.4
World total[b] (90)	**6.7–19.1**	**12.4**	**4.4**
Least developed regions (74.5)	**1.9–6.1**	4.2	5
Less developed regions (93)	**6.3–20.9**	14.6	5.1
More developed regions (98.9)	**14.5–27.2**	12.7	2.6

Table 2. Change in cesarean section rates in 121 countries categorized according to the United Nations (UN) geographical grouping from 1990 to 2014[a]. Changes are presented as absolute increase and relative increase as average annual rate of increase (AARI). [a] If the data in 2014 is not available, the latest data is used instead. If the data in 1990 is not available, the earlier data from 1985-1995 is used. [b] Countries categorized according to the UN geographical grouping. Number of live births in 2000 was used as a weight to calculate the regional coverage. [c] AARI: average annual rate increase = $(a_m/a_n)^{[1/(n-m)]-1}$. a_m: the first observation of C-section rate, a_n: the latest observation of C-section rate, m: the first observed year, n: the latest observed year. [d] estimates for subregions with a coverage less than 60% are not calculated. Coverage for Middle Africa is 29% and for Southern Africa is 4.6%. Reprinted from "The Increasing Trend in Cesarean Section Rates: Global, Regional and National Estimates: 1990-2014", by A.P. Betrán et al., 2016, *PLoS One*, 11(2):e0148343.

The lowest rates of C-section are found in Africa (7.4%) while the highest rates are found in Latin America and the Caribbean (42.2%). In each region, the countries with the highest C-section rate were: Brazil (55.6%) and Dominican Republic (56.4%) in Latin America and the Caribbean; Egypt (51.8%) in Africa; Iran (47.9%) and Turkey (47.5%) in Asia; Italy (38.1%) in Europe; United States (32.8%) in Northern America; and New Zealand (33.4%) in Oceania (Figure 5).

These rates are in disagreement with the International Healthcare Community which considers the ideal rate for C-section to decrease mortality in women and their babies to be between 10 and 15%[169]. Numerous factors have been associated to this rise in C-section rates. Indeed, medical practice regarding C-section delivery has changed due to the (1) decrease in vaginal births after a C-section delivery, (2) increase in maternal request, (3) increase in maternal age, and (4) changes in clinical decision making (with a higher rate of C-section delivery in private compared to public facilities)[170–172].

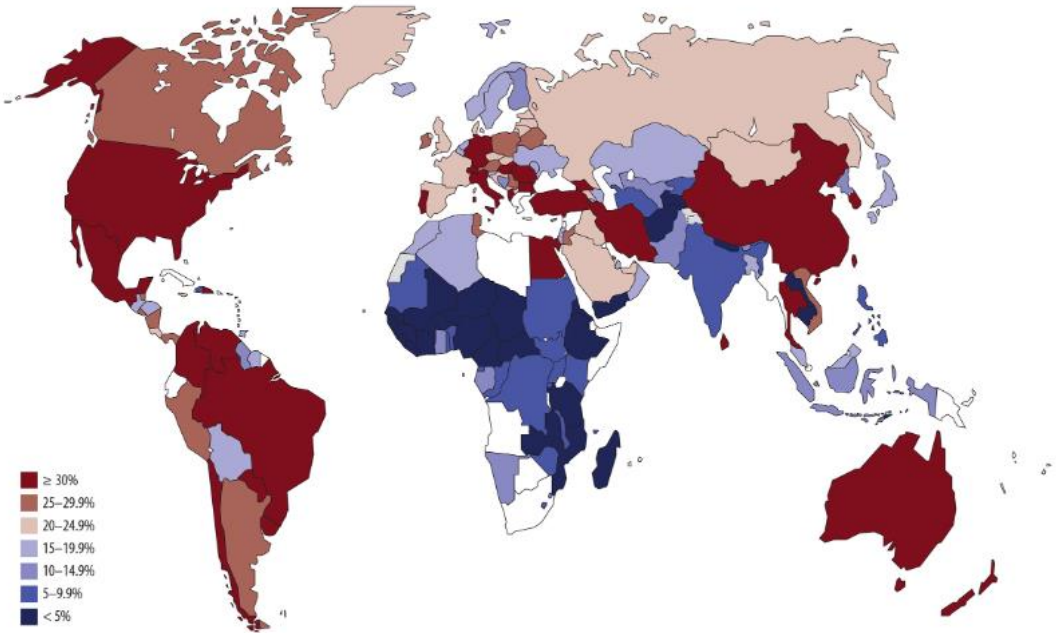


Figure 5. Latest available data on cesarean section rates by country. Reprinted from "The Increasing Trend in Cesarean Section Rates: Global, Regional and National Estimates: 1990-2014", by A.P. Betrán et al., 2016, *PLoS One*, 11(2): e0148343.

2.3. Classification

C-section has been used for decades when natural birth was impossible. With time, this led to C-section being performed under two different circumstances, either as an emergency or elective (planned).

2.3.1. Emergency C-section

Emergency C-section is a C-section performed due to an "immediate threat to life of woman or fetus"[173] that can be done either at term or preterm, and with or without labor. This rescue procedure, accounting for 3% of all performed C-sections[174], is recommended to be performed in a 30-minute interval between decision to operate due to an emergency and the incision[175]. However, definition of "what an emergency is" is hard to tackle. Indeed, a variety of symptoms can lead to an emergency C-section, depending on their acuity and severity. This includes hemorrhage from placenta previa, abruptio placentae, prolapse of the umbilical cord, uterine rupture, fetal distress and prolonged labor.

2.3.2. Elective C-section

The important question before performing an elective C-section is whether a planned C-section is more beneficial than harmful to a woman and her baby than vaginal birth. However, a growing number of women request elective C-section delivery without any medical indications

due to the general perception that C-section delivery is much safer than in the past. Yet, C-section delivery is a major surgery implying risks for complications.

2.3.2.1. With medical indication

Doctors recommend elective C-section to their patient when the risks for vaginal delivery are higher than those for C-section. Medical indications consist of women- and/or fetal- related complications. Women-related complications comprise: cephalopelvic disproportion, previous C-section, infections (e.g. HIV, genital herpes), placental disorder (e.g. placenta previa), multiple pregnancy and chronic health conditions (e.g. obesity, diabetes, high blood pressure, heart disease). Fetus-related complications include: breech presentation and birth defects such as a congenital heart disease.

2.3.2.2. On maternal request

C-section delivery on maternal request is described as a primary prelabor surgery in the absence of any maternal or fetal indications. Even though C-section delivery was first used to reduce the risks for the mother and the fetus, nowadays, it is also perceived as a way to escape from labor pain. The American College of Obstetricians and Gynecologists reported the risk and benefits of having a C-section on maternal request for the maternal and fetal sides.

On the maternal side, benefits comprise a decreased risk of postpartum hemorrhage, transfusion, and surgical complications, as well as a decrease in urinary incontinence during the first year after delivery. Risks include longer hospital stay, greater complications in future pregnancies (e.g. uterine rupture, placenta previa, placenta accreta, bladder and bowel injuries, need for hysterectomy), increased risk of postpartum cardiac arrest, wound hematoma, puerperal infection, anesthetics complications, and venous thromboembolism and hemorrhage requiring hysterectomy[176–178] (Table 3).

On the fetal side, benefits include a lower risk of neonatal encephalopathy and asphyxia[179], brachial plexus injury, and fetal laceration at the time of the C-section compared to an emergency C-section[180]. Risks consist of respiratory morbidity (e.g. transient tachypnea, persistent pulmonary hypertension)[181], and complications relative to prematurity since C-sections are usually done before term (e.g. hypothermia, hypoglycemia, neonatal intensive care unit admissions)[176].

	Complications of delivery by C-section on the mother
Intraoperative	Infections
	Organ injury (bladder, intestines, ureter, etc.)
	Anesthetics complications
	Blood loss
	Blood clots
	Hysterectomy (e.g. due to a hemorrhage)
Postoperative	Thromboembolic complications
	Uterine adhesion
	Persistent pain
	Postpartum cardiac arrest
	Wound hematoma
	Puerperal infection
Risks for subsequent pregnancies	Intrauterine growth retardation and preterm delivery
	Spontaneous abortion
	Ectopic pregnancy
	Stillbirth
	Uterine rupture
	Infertility
	Placenta previa
	Placenta accreta

Table 3. Complications of delivery by C-section. Adapted from "Indications for and Risks of Elective Cesarean Section", by I. Mylonas et al., 2015, *Deutsches Ärzteblatt International*, 112(29-30): 489-495.

2.4. Divergences in parturition

As described earlier, C-section delivery is a surgical procedure that is performed either as an emergency or recommended when the risks to give birth are higher after vaginal than C-section delivery. Because of the diversity of factors associated to C-section delivery, including gestational age at the time of C-section and presence or not of labor, the impact of C-section on the development of the newborn must be taken with caution.

2.4.1. Maternal divergences

Little is known about the physiological changes of C-section compared to vaginal delivery in the mother.

2.4.1.1. Uterine contraction

Although uterine contractions are present during emergency C-section with labor, this is not the case for elective C-section. This absence of labor is associated with hormonal changes. In human parturition, uterine contraction during labor is associated with a change in the ratio

PRA/PRB in favor of PRA in the myometrium. In pregnant women with no sign of labor (as for elective C-section), this PRA/PRB ratio is maintained at 0.5, inducing the uterine quiescence described previously[182].

2.4.1.2. Hormonal levels

Even though catecholamines levels in mothers going under elective C-section are higher than those of resting adults, there are still lower than those seen after vaginal deliveries. This is partially due to the absence of pain associated with uterine contractions and effort during labor which are known factors triggering the release of catecholamines[183]. Furthermore, anesthesia impacts this release since catecholamines levels were found to be lower in women receiving epidural anesthesia compared to general anesthesia when undergoing an elective C-section[183]. As described before, catecholamines are necessary for neonatal adaptation, and their lower release in elective C-section might be disadvantageous in terms of neonatal adaptation[183].

ACTH, cortisol and β-endorphins are also lower after elective C-section than after vaginal delivery with or without epidural anesthesia, while prolactin levels are higher[184]. Therefore, the concentration of stress-associated hormones overall is lower after elective C-section in the mother and might further impact neonatal adaptation at birth.

2.4.2. Fetal divergences

C-section delivery, and particularly elective C-section between 37 and 40 weeks of gestation, is generally believed to be harmless. Yet, it still affects the fetal adaptation from intrauterine to extrauterine life.

2.4.2.1. Anesthesia

In elective C-section deliveries, either general or epidural-spinal anesthesia can be used. General anesthesia is a medically induced coma producing anesthesia of the entire body, with loss of consciousness. It is preferably done in women in emergency situations (e.g. prolapse of umbilical cord, fetal distress, etc.) or when regional anesthesia is contraindicated (e.g. coagulopathy, infections, bleeding). Regional anesthesia consists in the removal of neural conduction and pain sensation in specific regions of the body without causing any loss of consciousness. In recent years, it has been preferably used due to its advantages including consciousness of the mother, no risks of aspiration, no respiratory depression in newborns, and no uterine atony[185].

Contrary to babies born under regional anesthesia, babies born under general anesthesia are more prone to have acidemia (low blood pH) and hypercapnia (abnormally elevated carbon dioxide)[186]. Even though some perinatal factors are dependent on the choice of anesthetics, others such as transient tachypnea of the newborn are not affected by this choice[185].

2.4.2.2. Fetal HPA axis

The mode of delivery affects subsequent stress responses, therefore modifying hormonal responses of the fetal HPA axis at birth and influencing the further development of the newborn.

Due to the absence of the mechanical trauma that is labor, as well as fetal hypoxia that might happen during uterine contractions, babies born by elective C-section have much lower catecholamines levels than those born vaginally and by emergency C-section[187]. Interestingly, anesthetics also impact fetal catecholamines levels as higher levels are found in infants born under epidural anesthesia compared to general anesthesia. This might be explained by the fetal exposition to intrauterine surgical stimulation which is longer in babies born under epidural anesthesia than by general anesthesia[183]. Furthermore, the use of enflurane as a general anesthetic has been shown to decrease the release of adrenal catecholamines[188].

Similarly, cortisol levels are decreased in elective C-section compared to vaginal delivery[189]. As described earlier, cortisol facilitates metabolic adaptation in response to the stress of being born by promoting the synthesis of thyroid hormones and the maturation of hepatic glucose metabolism enzymes among other things. Moreover, infants with high cortisol levels at birth are more alert in the first few hours of birth than those with low cortisol levels[190]. This suggests that infants born by elective C-section might be less alert at birth, and therefore less available for social interaction and breastfeeding seeking behaviors.

Alterations in these stress-associated hormonal levels are correlated with impaired cerebral development in rats[191] and central nervous system disorders in humans[192]. Furthermore, the low activity of the HPA axis in infants born by elective C-section might delay neonatal adaptation by insufficiently increasing cardiac performance, gluconeogenesis, lung adaptation, and thermogenesis at birth. This hypothesis is supported by findings that C-section born infants, compared to infants born vaginally, have hypoglycemia before two hours of age and lower free fatty acids and C-peptide (linker between the A- and B- chains of insulin) levels persisting at two hours of age[121].

2.4.2.3. Respiratory adaptation

Even though inspiratory volumes in infants born by elective C-section are similar to those in vaginal infants, expiratory pressures are smaller and functional residual capacity is absent in these babies[193]. These lung changes result in C-section-delivered infants at any gestational age having a higher incidence of respiratory distress[194], as well as transient tachypnea, smaller thoracic gas volume, higher total pulmonary resistance[195], and persistent pulmonary hypertension[93] than infants born vaginally. Moreover, respiratory morbidity is related to iatrogenic prematurity as the earlier the gestational age at the time of the elective C-section, the higher the morbidity rate for respiratory distress[93].

This is mainly due to the absence of mechanical compression (vaginal squeeze) at birth that is known to induce increased HPA axis activity[97]. In infants born by elective C-section, the low HPA axis activity might result in delayed absorption of lung liquid and reduced release of surfactant[183]. In addition, epithelial Na^+ channels involved in the absorption of lung fluid are developmentally regulated and their peak expression is achieved at term[196]. Therefore, children born by elective C-section before term might be born with a lower expression of Na^+ channels which reduces their ability to clear fetal lung fluid at birth, as seen in mice[197]. Finally, children born by elective C-section might present immaturity of lung epithelial transport, as seen in human neonates suffering from transient tachypnea[198].

2.4.2.4. Cardiac adaptation

Studies have shown an alteration in cardiac adaptation in infants born by elective C-section compared to vaginally delivered infants. Indeed, C-section has been associated to decreased left ventricular output at birth[199,200]. In animal studies, the increase in circulating catecholamines levels is critical for postnatal cardiac output and blood organ distribution adaptations at birth[201,202]. Yet, the capacity to adapt to cardiovascular changes at birth has been shown to be similar for infants born by elective C-section or vaginally delivered[203].

The heterogeneity of results might be due to the choice of anesthetics during the surgical procedure, as they are known to affect differently catecholamines levels. In humans, left ventricular output (seen with the isovolumic contraction time) was shorter after epidural anesthesia compared to general anesthesia[200]. On the contrary, epidural and spinal anesthesia influence neonatal left ventricular adaptation and timing of ductus constriction in a similar way[204]. Yet, regional anesthetics are not harmless, as shown with spinal anesthesia that can lead to major effects such as decreased systemic vascular resistance and increased heart rate and

stroke volume[205], as well as increased cardiac output right after anesthesia injection and delivery[206]. Moreover, animal studies showed that increasing concentrations of bupivacaine (local anesthetic) resulted in increased depressant effect on cardiac conductivity and contractility[207,208].

2.4.2.5. Gut microbiota

In vaginally delivered infants, the microbiome resembles that of the mother's vaginal and feces[112], and is composed of beneficial bacteria for the development of the immune system such as *Bifidobacterium longum* subspecies *infantis*, *bacteroidetes* and *lactobacilli*[209,210]. In contrast, in infants born by elective C-section, the microbiome at birth harbors no vaginal microbes and is more similar to the mother's skin, hence being constituted of bacteria such as *Staphylococcus* and *Corynebacterium*[112]. Furthermore, intestinal postnatal colonization by *Bacteroides* and *Bifidobacterium* subspecies is delayed[211] and *Clostridium difficile* levels are higher than in babies born vaginally[212]. Interestingly, differences in microbial composition after C-section delivery persist through life and might be associated with long-term deleterious effects[213,214]. Indeed, the gut-brain axis is known to influence brain development and behavior[215], and its alteration in the initial months of life could lead to the subsequent emergence of adverse mental health outcomes.

2.5. Long-term consequences

As C-section delivery has short-term effects on neonatal adaptation, this surgical procedure is also associated with long-term adverse consequences.

2.5.1. Autoimmune diseases

Autoimmune diseases are conditions in which immune responses are abnormal and result in the immune system attacking its own body. C-section delivery has been associated with an increased risk of developing autoimmune diseases.

Indeed, infants born by elective, but not emergency C-section, have an increased risk of developing celiac disease[216,217]. Because of the positive association with elective but not emergency C-section, it is hypothesized that the fetal microbiome plays a role in the development of celiac disease. Nonetheless, other factors deviating between elective and emergency C-section (e.g. prenatal stress and hormones associated to labor) might also alter the neonate neuronal development.

The risk of developing childhood-onset type-1 diabetes is also increased by 20% in children born by C-section compared to those born vaginally[218]. Changes in microbiota due to C-section delivery have been proposed as a possible explanation to this association since microbiota is known to influence the development of the immune system[219].

2.5.2. Obesity

Similar to celiac disease, C-section delivery is associated with an increased risk of obesity[220,221], as seen with higher body mass by 6 weeks of age and persisting at age 15 compared to vaginal born infants.

2.5.3. Asthma and other allergic diseases

C-section delivery is controversially associated with higher risks to develop asthma in childhood and adulthood[222–224]. It is noteworthy that one recent paper showed that C-section delivery differentially increases the risk of developing asthma. In this paper, neonates born by C-section who experienced respiratory distress syndrome or transient tachypnea at birth have a higher risk to develop asthma during childhood. On the contrary adolescents at the age of 15 born by C-section do not have an increased risk to develop asthma, nor impaired lung function[225]. Even though risks to develop asthma might be increased in children born by C-section, no such effects are observed for wheezing, atopy, hay fever, and atopic eczema[222,224]. However, children born by C-section have a higher risk of sensitization to any specific allergen at the age of 8, and those born from at least one allergic parent have aggravated risks of asthma[226].

The development of asthma might also be linked to the disruption of the microbiota, as observed in children born by C-section, that may prolong immunological immaturity. Indeed, asthmatic children have more proteobacteria and less *Bacteroidetes* than non-asthmatic children[227], a feature that is also found in infants born by C-section[211].

Nonetheless, we must remember that genetics are also likely to play a role, as suggested by the higher susceptibility to develop asthma in children born from allergic parents. Indeed, microbes colonizing the gut and lungs might be dependent on the individual gene expression in those organs. Furthermore, the environment also influences the risk to develop asthma, as children raised in farms are less prone to develop asthma and allergies than others[227].

2.5.4. Neurodevelopmental disorders

Epidemiological studies report an increased risk for children born by C-section delivery to have neurodevelopmental disorders, yet the correlation between these two remains controversial. Moreover, gestational age impacts the development of brain disorders as children born by C-section at gestational ages prior to 38 weeks are associated with more neurodevelopmental disorders, and the risk of developing these disorders decreases as gestational age increases[228].

2.5.4.1. Obsessive-Compulsive Disorder (OCD)

OCD is a chronic and long-lasting disorder in which a person has symptoms of obsessions and compulsions. Obsessions consist of repeated thoughts and urges that cause anxiety, while compulsions consist of repetitive behaviors in response to the obsessions.

OCD is a multifactorial disease with genetic and environmental factors increasing the risk of developing it. An epidemiological study reported that C-section is one of the perinatal factors associated with an increased risk of developing OCD, and that gestational age and the risk of developing OCD are inversely correlated[229]. However, this study does not specify if the C-section was performed as an emergency or elective, hence, this remains to be further evaluated.

2.5.4.2. Tourette Syndrome (TS) and Chronic Tic Disorders (CTD)

The DSM-IV divides tic disorders into TS and CTD. TS is a neuropsychiatric disorder which manifests usually during childhood. It is characterized by repeated involuntary movements (motor tics) and uncontrollable vocal sounds (vocal tics). CTD, contrary to TS, is characterized by the manifestation of either a motor or a vocal tic, but not both.

Epidemiological studies report that C-section delivery increases the risk of developing TS and CTD[230,231]. However, these studies do not specify the risk probability between emergency *vs*. elective C-section, nor if the risk evolves in parallel to the gestational age.

2.5.4.3. Bipolar Disorder (BD)

BD, formerly called manic-depressive illness, is a disorder that is characterized by extreme mood swings, and unusual shifts in energy and activity levels.

Two epidemiological studies report an increased risk of developing BD in children born by C-section. Interestingly, emergency and planned C-section were compared, and the risk to develop BD was only increased in children born by elective C-section[232,233].

2.5.4.4. Dopamine-related disorders

Numerous dopamine-related disorders are referenced in epidemiological studies, but we will discuss only Attention-Deficit Hyperactivity Disorder (ADHD) and schizophrenia.

ADHD is a disorder which manifests during childhood and is characterized by difficulties for a person to pay attention and control impulsive behaviors. Schizophrenia is a chronic and severe mental disorder that affects a person's perception of its environment and oneself. The symptoms are divided into three categories: positive, where people may "lose touch" with reality (e.g. hallucinations); negative, associated with a disruption of normal emotions and behaviors; and cognitive, with changes in memory or other aspects of thinking.

Epidemiological studies have correlated C-section delivery with an increased risk of ADHD, even though they did not differentiate between emergency and elective C-section[234,235]. On the other hand, epidemiological studies reported controversial results on the link between C-section and schizophrenia. Nonetheless, in these studies, the risk of developing schizophrenia was never affected by elective C-section, but instead by emergency C-section in which fetal hypoxia or anoxia is common[233,236].

Further exploration revealed that schizophrenic patients born by C-section delivery have lower C-reactive protein levels (a protein which levels increase in response to inflammation), and lower premorbid intellectual ability[237]. Moreover, preclinical data in rats report that C-section delivery with or without anoxia (mimicking fetal anoxia found in humans) induces long-term changes in brain dopaminergic pathways, a key feature in schizophrenia and ADHD (Table 4 and Table 5).

		Changes relative to vaginally born controls
AMPH-induced locomotion	dose 0.5mg/Kg[238] dose 3mg/Kg[239] dose 5mg/Kg[239]	↑ ↑ 20 and 30 min after AMPH injection No change
Locomotor activity after injection of Drd1 and Drd2 agonist[239]		↑
Norepinephrine levels[240]		↓ AM in males, ↑ VH/Th in females, ↑ Th in females in estrus
Dopamine levels[240,241]		↓ PFC ↑ Nacc/Str, ↑ Nacc/Str in males
Dopamine transporter binding[242,243]		↑ Str/Nacc
Drd1-like dopamine receptors binding[239,244]	2 weeks old 1 month old 3-4 months old	No change No change ↑ Nacc/OTb/IL cx/AON
bFGF (a dopamine trophic factor)levels[245]	2 weeks old 3.5 months old	No change
Tyrosine hydroxylase mRNA levels[246]		↑ Nacc
Insulin-like growth factor I receptors binding[247]		↑ CA1/CA2/CA3/DG/MolCm ↑ in all HPC layers
Insulin-like growth factor II receptors binding[247]		No change
AMPA receptors binding[248]		↑ Nacc shell
NMDA receptors binding[248]		↑ Cg cx
Kainate receptors binding[248]		↑ CA1
Dendritic length of pyramidal neurons (PFC, CA1) and medium spiny neurons (Nacc)[249]	P14 P21 P35	↓ PFC ↑ PFC ↑ PFC, ↑ Nacc
Dendritic arborization of pyramidal neurons (PFC, CA1) and medium spiny neurons (Nacc)[249]	P14 P21 P35	↓ PFC ↑ PFC ↑ PFC, ↑ Nacc
Dendritic spine density of pyramidal neurons (PFC, CA1) and medium spiny neurons (Nacc)[249]	P2 P7 P14 P21 P35 P70	No change No change No change ↓ CA1 ↓ PFC, ↓ CA1 ↓ PFC

Table 4. Summary of changes in dopaminergic parameters in rats born by C-section in comparison to vaginally born controls. AON: anterior olfactory nucleus, AM: amygdala, AMPH: amphetamine, bFGF: basic fibroblast growth factor, Cg cx: cingulate cortex, DG: dentate gyrus, Drd1: dopamine receptor D1, Drd2: dopamine receptor D2, IL cx: infralimbic cortex, MolCm: molecular cell layer of cerebellum, Nacc: nucleus accumbens, OTb: olfactive tubercle, PFC: prefrontal cortex, Str: striatum, Th: thalamus, VH: ventral hippocampus. Adapted from "Birth insult interacts with stress at adulthood to alter dopaminergic function in animal models: possible implications for schizophrenia and other disorders", by Boksa et al., *Neuroscience and Biobehavioral Reviews*, 27 (2003): 91-101.

		Changes relative to vaginally born controls
AMPH-induced locomotion	dose 0.5mg/Kg[238] dose 3mg/Kg[239] dose 5mg/Kg[239]	↑ - -
Locomotor activity after injection of Drd1 and Drd2 agonist[239]		-
Norepinephrine levels[240]		↓ Nacc in females in estrus
Dopamine levels[240,241]		No change
Dopamine transporter binding[242,243]		↑ Cg cx/IL cx
Drd1-like dopamine receptors binding[239,244]	2 weeks old 1 month old 3-4 months old	- - No change
bFGF (a dopamine trophic factor) levels[245]	2 weeks old 3.5 months old	↓ VTA/SNc ↓ VTA, ↑ NAcc
Tyrosine hydroxylase mRNA levels[246]		No change
Insulin-like growth factor I receptors binding[247]		↑ CA1/CA2/CA3/DG ↑ in all HPC layers
Insulin-like growth factor II receptors binding[247]		↑ CA1/CA2/CA3/DG ↑ in all HPC layers
AMPA receptors binding[248]		No change
NMDA receptors binding[248]		↑ AON
Kainate receptors binding[248]		↑ CA1
Dendritic length of pyramidal neurons (PFC, CA1) and medium spiny neurons (Nacc)[249]	P14 P21 P35	↓ CA1 ↓ PFC, ↓ CA1 ↓ CA1
Dendritic arborization of pyramidal neurons (PFC, CA1) and medium spiny neurons (Nacc)[249]	P14 P21 P35	↓ CA1 ↓ CA1 ↓ CA1
Dendritic spine density of pyramidal neurons (PFC, CA1) and medium spiny neurons (Nacc)[249]	P2 P7 P14 P21 P35 P70	↓ PFC, ↓CA1 ↓ PFC ↓ PFC, ↓CA1 ↓ PFC, ↑ CA1 ↓ PFC, ↑ CA1, ↑ Nacc No change

Table 5. Summary of changes in dopaminergic parameters in rats born by C-section + anoxia in comparison to vaginally born controls. AON: anterior olfactory nucleus, AMPH: amphetamine, bFGF: basic fibroblast growth factor, Cg cx: cingulate cortex, DG: dentate gyrus, Drd1: dopamine receptor D1, Drd2: dopamine receptor D2, HPC: hippocampus, IL cx: infralimbic cortex, Nacc: nucleus accumbens, PFC: prefrontal cortex, SNc: substantia nigra pars compacta, VTA: ventral tegmental area. Adapted from "Birth insult interacts with stress at adulthood to alter dopaminergic function in animal models: possible implications for schizophrenia and other disorders", by Boksa et al., *Neuroscience and Biobehavioral Reviews*, 27 (2003): 91-101.

2.5.4.5. Autism Spectrum Disorders (ASD)

ASD is a developmental disorder characterized by communication and behavioral alterations. Epidemiological studies suggest that C-section delivery might lead to a higher incidence of ASD[250–252], yet, this has been disputed by other investigations[253,254]. Interestingly, the risk of developing ASD has been shown to be similar for children born by emergency or elective C-section across gestational ages[252]. Moreover, children born by C-section between 36 to 42 weeks of gestation have a significant risk of developing ASD, but this risk is consistent across ages. However, due to the variability and low number of children born by C-section before 36 weeks of gestation, the relationship to ASD remains debatable[252].

My thesis work aimed at further understanding if C-section delivery had an influence on the risk of developing ASD, if this risk was different according to the gestational age when the C-section was performed, and the biological characteristics that might be associated to C-section delivery and ASD.

3. AUTISM SPECTRUM DISORDERS (ASD)

Since its initial description in 1944 by Leo Kanner[255], the criteria by which a patient is diagnosed with ASD has evolved. At present, ASD are known as a group of complex neurodevelopmental disorders characterized by three main core symptoms: persistent deficits in social communication and social interactions, deficits in nonverbal communicative behaviors, and restrictive and stereotypic behaviors[256]. The severity of deficits in social communication and interactions range from failure of normal reciprocal conversation to failure to initiate or respond to social interactions. For nonverbal communicative behaviors, the severity ranges from poorly integrated nonverbal communication to total lack of facial expressions and nonverbal communication. For stereotypic behaviors, they vary from stereotyped or repetitive motor movements or speech, to hyper- or hypo-reactivity to sensory inputs of the environment. Depending on the severity and number of symptoms present, a person is diagnosed for one of the numerous pathologies encompassed in the designation of ASD: autistic disorder, Asperger's syndrome, pervasive developmental disorder (PDD) not otherwise specified, childhood disintegrative disorders, social pragmatic communication disorder, and Rett syndrome.

The time of appearance of symptoms typically happens in the first 3 years of life and persist to adolescence and adulthood, but this view has been recently tackled as it is dependent on arbitrary factors such as the parents' acknowledgment of their child's symptoms and the absence of earlier specific signs[257,258]. However, it is generally accepted that the time of onset of this disorder is much earlier than the time of appearance of symptoms. In addition, children with ASD can also have other comorbidities including epilepsy, anxiety, ADHD, BP, clinical depression, Down syndrome, Fragile X syndrome, OCD, TS, tuberous sclerosis, gastrointestinal symptoms, immune disorders, macro- or micro-cephaly, and sensory sensitivities[259–265].

3.1. Epidemiology

For the past decades, the prevalence of ASD has increased worldwide and is now thought to range between 0.01 and 2% with a median prevalence estimated around 0.62%[266], and affecting males more than females. This increase in the prevalence of ASD is in part due to the increased recognition, understanding, and awareness of these disorders. Furthermore, changes in clinical practice and criteria used to diagnose ASD have evolved for the past years and we now refer to either the DSM-V (Diagnostic and Statistical Manual of Mental Disorders) or the ICD-10

(International Statistical Classification of Diseases and Related Health Problems) to diagnose a person. However, these changes in recognition are not the sole factor accounting for this increased prevalence. Indeed, ASD are multifactorial disorders resulting from genetic and environmental risk factors.

3.2. Etiology

3.2.1. Genetic factors

The identification of genes and chromosomal anomalies implicated in ASD has increased over the past years and is now believed to account for 10-25% of children with ASD[267,268]. Genetic mutations, which alter developmental pathways of neuronal and axonal structures involved in synaptogenesis, are preferentially observed in genes likely to be haploinsufficient[269]. Furthermore, these alterations appear to be strongest in cortical neurons (especially pyramidal projection neurons), cerebellar granule cells, and the medium spiny neurons of the striatum[270]. These genes' location and function are referenced and summarized on Table 6.

Genes related with ASD	Location	Role
ADNP	20q13.13	Regulation of gene activity
ANK2	4q25-q26	Regulation of cell cytoskeleton
ARID1B	6q25.3	Regulation of gene activity by promoting SWI/SNF protein complexes
ASH1L	1q22	Regulation of histone configuration and chromosome shaping
ASMT	Xq22.33/Yp11.2	Promote synthesis of melatonin
ASXL3	18q11	Regulation of gene transcription
CHD8	14q11.2	Regulation of gene activity
CNTNAP2	7q35-36	Regulation of radial and longitudinal organization of myelinated axons
DYRK1A	21q22.13	Regulation of neuronal proliferation. differentiation. plasticity and death
GABRB3	15q12	Mediate cellular responses to histamine Component of GABA receptor
GRIN2B	12p13.1	Subunit of the NMDA receptor ion channel
IQSEC3	12p13.33	Act as a guanine nucleotide exchange factor
KATNAL2	18q21.1	Promote rapid reorganization of cellular microtubule arrays
NLGN3	Xq13.1	Regulation of synapse function and signal transmission
NRNX1	2q16.3	Regulation of the formation of intracellular junctions Function as receptors and cell adhesion molecules in the nervous system
NRNX2	11q13.1	
NRNX3	14q24.3-31.1	
POGZ	1q21.1	Regulation of gene transcription and activity
PRRT2	16p11.2	Interaction with SNAP25. a protein involved in neuronal signaling
PTEN	10q23	Regulation of cell division
SCN2A	2q24.3	Subunit of the voltage-gated sodium channels
SETD5	3p25.3	Probable transcriptional regulator

SHANK1		19q13.33	Scaffold protein in postsynaptic density of excitatory synapses
SHANK2		11q13.3-13.4	
SHANK3		22q13.33	
SUV420H1		11q13.2	Function has not been determined yet
SYNGAP1		6p21.3	Regulation of synapse adaptations and promoting proper brain wiring
TBR1		2q24.2	Transcriptional regulator in developmental processes
Genes	**Comorbidity**	**Location**	**Role**
FMR1	Fragile X	Xq27.3	Regulate mRNA trafficking from the nucleus to the cytoplasm
MECP2	Rett Syndrome	Xq28	Mediate transcriptional repression
TSC1	Tuberous Sclerosis	9q34.13	Negative regulation of mTOR signaling Act as a tumor suppressor
TSC2	Tuberous Sclerosis	16p13.3	
NF1	Neurofibromatosis	17q11.2	Negative regulator of signal transduction pathway
PAH	Phenylketonuria	12q23.2	Rate limiting enzyme in phenylalanine catabolism
-	Down syndrome	21	3 copies of the chromosome instead of 2
GRIK2	Huntington Disease	6q16.3	Subunit of the ionotropic kainate receptor
RELN	Alzheimer Disease	7q22.1	Regulation of layering of neurons in cerebral cortex and cerebellum Regulation of microtubule function in neurons and neuronal migration
UBE3A	Angelman Syndrome	15q11.2-13	Belong to the ubiquitin protein degradation system
MAGEL2	Prader-Will Syndrome	15q11.2	Regulator of retrograde transport and circadian clock Promote the cytoplasmic accumulation of CLOCK

Table 6. ASD-related genes, their chromosomal location and role. Adapted from "A Short Review on the Current Understanding of Autism Spectrum Disorders", by H.R. Park et al., 2016, *Experimental Neurobiology*, 25(1): 1-13.

3.2.2. Environmental factors

Although ASD are thought to develop due to a high vulnerability to genetic mutations, environmental factors also contribute to their development. Indeed, it has been suggested that ASD may be a disease already present in utero in response to environmental exposures during pregnancy. Environmental factors can be divided into prenatal, perinatal, and postnatal factors.

3.2.2.1. Prenatal factors

Prenatal factors contributing to the increased risk of developing ASD are the exposure to teratogens, maternal infections, maternal nutrition, maternal stress, and chemical exposure (Table 7).

Teratogens associated with an increased incidence of ASD include valproic acid (VPA), ethanol, thalidomide, misoprostol and cigarette[271,272]. VPA is a medication that was used to treat epilepsy by increasing GABA concentrations in the brain[273]. Its teratogenic effects during pregnancy may be related to a deficiency in folic acid, induction of oxidative stress, and inhibition of histone deacetylases[274]. Ethanol exposure in pregnant women, whose incidence

has increased in many countries, deregulates cell proliferation, migration, and apoptosis, in addition to epigenetically modifying gene expression[275,276]. Thalidomide, a drug which was prescribed to treat morning sickness in pregnant women, has been reported to cause congenital malformations. Its deleterious effects are due to its anti-angiogenic action and possibly its role of inhibition of cell migration by down-regulating adhesion receptors and interacting directly with DNA[277]. Exposure to misoprostol, a drug used in pregnancy to decrease the risk of gastric ulcer, induces congenital defects because of the temporary vascular disruption in the placental-fetal unit[278]. Finally, cigarette smoking during pregnancy causes multifactorial adverse effects such as blood flow restriction to the placenta and reduced birth weight[279].

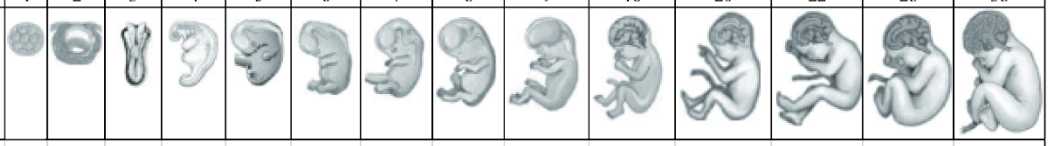
Trimester	First									Second			Third	
Gestation Weeks	1	2	3	4	5	6	7	8	9	16	20	22	28	38
														
Exposure														
Valproic Acid				Days 22-28										
Ethanol			Weeks 3-5											
Thalidomide			Days 20-24											
Misoprostol						Week 6								
Cigarette	1st, 2nd, and 3rd trimesters													
Viral infection	1st trimester													
Bacterial infection										2nd trimester				
Omega-3 fatty acid deficiency	1st, 2nd, and 3rd trimesters													
Vitamin A deficiency	1st, 2nd, and 3rd trimesters													
Vitamin D deficiency	1st, 2nd, and 3rd trimesters													
Maternal stressors												Weeks 25-28		
Air pollution	1st, 2nd, and 3rd trimesters													
Pesticides				Days 26-81										

Table 7. Critical periods of susceptibility for ASD. Adapted from "Maternal lifestyle and environmental risk factors for autism spectrum disorders", by K. Lyall et al., 2014, *International Journal of Epidemiology*, 43(2): 443-464.

Maternal infections during pregnancy, also called maternal immune activations (MIA), have also been reported to increase the risk of developing ASD[280,281]. Interestingly, preclinical and clinical studies have identified that the critical period of exposure to infections and to develop MIA is different for virus and bacteria, and correspond to the first and second trimester respectively[282]. Nonetheless, it is important to note that several studies (reviewed in [281]) have shown no correlation between MIA and the risk of developing ASD.

Maternal nutrition also influences the risk of developing ASD, by either increasing or decreasing it. Indeed, folic acid intake during pregnancy might decrease the risk of developing ASD[283,284], while omega-3 fatty acids and vitamin D deficiencies increases this rate[285,286].

Maternal stress during pregnancy is considered a risk factor for adverse psychological outcomes such as schizophrenia, emotional disturbances, and ASD[287]. These stressors might alter neuronal development, as seen in preclinical studies where maternal stress alters the HPA axis and amygdala expansion in the offspring[288,289].

Chemicals increasing the risk of developing ASD include ambient air pollution[290], persistent organic pollutants, and pesticides. Ambient air pollution is a mixture of gases and particles that are either primarily emitted (e.g. from industrial processes, vehicular exhaust, combustion product, dust) or secondarily formed in the atmosphere. Some of these compounds are metals, styrene, methylene chloride, perchloroethylene, formaldehyde and others[290,291]. Furthermore, bioaccumulation of persistent organic pollutants including polychlorinated biphenyls and polybrominated diphenylethers also increases the risk of developing ASD[292]. Pesticides have also been reported to increase the risk of ASD, as a maternal residence near agricultural areas with pesticides increased the risk of ASD in children[293].

3.2.2.2. Perinatal factors

In addition to genetic and prenatal factors, perinatal factors have been related to an increased risk of developing ASD. These factors include breech presentation, low Apgar score at 5 minutes, prematurity, low birth weight, birth asphyxia, advanced maternal age, uterine bleeding, meconium staining, and C-section delivery (described in 2.6.4.5.)[253,269]:

- Breech presentation refers to the fetus in longitudinal lie with the buttocks/feet entering the pelvis first. Breech presentation occurs in 3 to 4% of all term pregnancies and the prevalence increases with earlier gestational ages[294].
- Apgar score is a method widely used to rapidly assess a newborn's health. This test consists in 5 categories (Appearance, Pulse, Grimace, Activity, Respiration) scaled from 0 to 2. Low scores at 5 min are associated with higher risks to develop ASD[295].
- Very low (<1500g) and moderately low (<2500g) birth weight are associated with a higher risk of developing ASD. Noteworthy, low birth weight specifically increases the risk of developing childhood autism and PDD, but not Asperger Syndrome[296].
- Meconium (composed of intestinal epithelial cells, amniotic fluid, lanugo, bile, water, and mucus) is normally stored in the infant's intestines during pregnancy. However, under pathological conditions (e.g. preeclampsia, placental insufficiency, smoking), meconium might be expelled in the amniotic fluid prior to birth. Hence, the fetus might be at risk of developing meconium aspiration syndrome where the baby at birth inhales

the contaminated fluid. Children exposed to meconium and with/without meconium aspiration syndrome have a higher risk to develop ASD compared to unexposed children[297].

3.2.2.3. Postnatal factors

Postnatal factors associated with an increased risk of developing ASD include infections, mercury toxicity, vitamin D deficiency, hyperbilirubinemia, chemical exposure, and gut dysbiosis:

- Postnatal infections such as meningitis, mumps, varicella, and ear infections are correlated with a higher risk of ASD during childhood[282]. This may be due to the fact that the brain still develops during the first years of life, therefore rendering it more susceptible to any insult than later in life.
- Sources of heavy metal poisoning include chemical products, fertilizers, industrial paint, building materials, fish that is high in mercury, silver dental fillings, mercury-containing preservatives (thiomersal) in vaccines, etc. The vast majority of epidemiological studies suggest that mercury poisoning increases the risk of ASD, possibly because of the neuroinflammation reactivity[298].
- In parallel to prenatal vitamin D deficiency, postnatal vitamin A and D deficiencies might also increase the risk of developing ASD[299]. One study reports a child with a vitamin D deficiency and suffering from ASD[300]. Moreover, ASD symptoms are improved after vitamin D supplementation, further supporting the role of vitamin D in the appearance of ASD[300].
- Hyperbilirubinemia, also called jaundice, results from high bilirubin production caused by an increased breakdown of fetal erythrocytes and a low hepatic excretory capacity. Similarly to previous postnatal infections and vitamin D deficiency, jaundice in the neonatal period has been shown to increase the risk of ASD[301].
- Chemical increasing the risk of developing ASD include bisphenol A[302], lead[303], and phthalates[304]. Early exposure to chemicals, via its effects on DNA methylation in the developing brain, might contribute to the deleterious neurodevelopmental outcomes[305]. Therefore, chemical exposure in genetically susceptible children might have a higher impact by altering their epigenome and modulating their developing brain.
- In patients with ASD, the gut microbiota which is known to influence brain development is altered. Indeed, their gut microbiota is less diverse than in nonautistic people and

presents non-exhaustively lower levels of *Bifidobacterium*[306] and higher levels of *Lactobacillus*[306], *Clostridium*[307] and *Bacteroidetes*[308].

3.3. Clinical and preclinical data

3.3.1. MRI studies

Clinical data mainly rely on magnetic resonance imaging (MRI) which allows the study of the brain in vivo in a non-invasive manner. Clinical studies have led to the theory of age-specific anatomic abnormalities in ASD, with overgrowth at early ages, and abnormal decline during adolescence and adulthood. These studies are summarized below (Figure 6, Table 8).

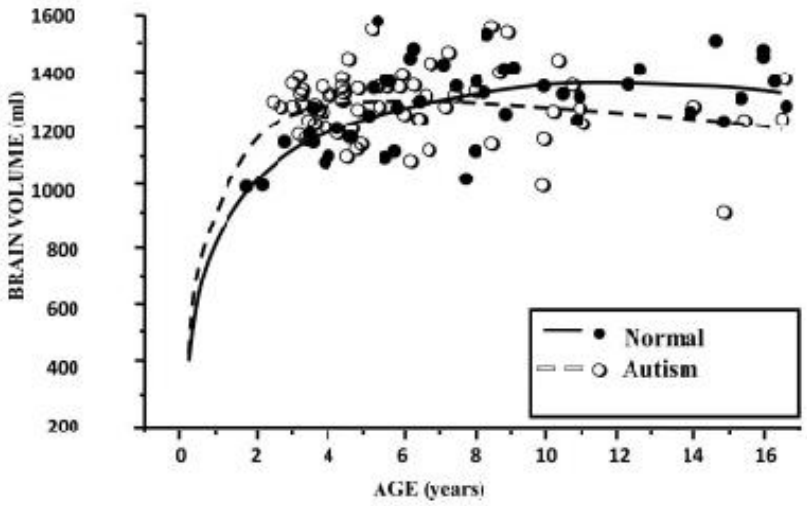


Figure 6. Brain growth in Autism children through 16 years of age. Data plots show individual MRI-based volumes in autistic 2-4 years old males as compared to the average volume in typical 2-4 years old males, and smaller overall brain volumes by 8-16 years of age. Reprinted from "Brain Growth Across the Life Span in Autism: Age-Specific Changes in Anatomical Pathology", by E. Courchesne et al., 2011, *Brain Research*, 1380: 138-145.

3.3.2. Biological markers

In parallel to MRI studies, doctors have tried to find specific biomarkers to enable the establishment of an earlier diagnostic of ASD. On this context, and in addition to clinical studies, preclinical studies enable us to further investigate the role of neurotransmitters and other factors in the pathology of ASD.

Age of patients in the study	Findings relative to control
18-35 months old	↑ total WM and GM volumes ↑ WM and GM volumes in frontal, temporal, and parietal-occipital lobes
2.5 years old	↑ in total WM and GM volumes
2-3 years old	↑ activity in response to forward speech in right frontal gyrus, right insula, and right postcentral gyrus
2.2-4 years old	↑ WM volume in frontal and parietal lobes, ↑ GM in frontal and temporal lobes
7-16 years old	↓ GM density in basal ganglia, inferior cerebellar vermis, posterior parietal cortices, left dorsolateral prefrontal cortex and left superior temporal cortex
Mean age of 9.73 years old	↑ GM volume in supramarginal gyrus, right postcentral gyrus, right medial frontal gyrus, and right posterior aspect of the cerebellum, ↓ GM volume in right parahippocampal gyrus ↓ WM volume in right anterior cingulate, and left superior parietal lobule
Mean age of 10.8 years old	↓ GM density in superior temporal sulcus ↓ WM density in right temporal pole and cerebellum
Mean age of 12 years old	↓ GM density in frontostriatal and parietal networks, in ventral and superior temporal gyrus ↑ WM density in cerebellum, left internal capsule and fornixes
Mean age of 13.2 years old	↑ GM volume in medial and dorsolateral frontal areas, in lateral and medial parts of temporal lobes, in parietal lobes, cerebellum, and claustrum ↓ WM volume in frontal, parietal, temporal, and occipital lobes
Mean age of 13.6 years old	↓ GM density in right inferior temporal gyrus, entorhinal cortex, right rostral tip of fusiform gyrus
15 years old	↑ GM density in posterior part of amygdala, ↓ GM density in anterior part of amygdala
16 years old	↑ GM volume in right fusiform and temporo-occipital gyrus, and left frontal pole ↓ GM volume in right thalamus
16 years old	↓ WM volume in corpus callosum, left middle temporal, right middle frontal, and left superior frontal gyri
Mean age of 19.2 years old	↑ GM density in midbrain, middle frontal gyrus, left reticular formation, right medial frontal gyrus, and left medial orbital frontal gyrus ↓ GM density in postcentral gyrus, ↓ WM in midbrain and left anterior cerebellum
Mean age of 21 years old	↑ GM volume in medial frontal gyri, left precentral gyrus, right post-central gyrus, right fusiform gyrus, caudate nuclei, and left hippocampus, ↓ GM volume in cerebellum
Mean age of 23.8 years old	↓ GM volume in right anterior and posterior insula, and right inferior frontal gyrus
Mean age of 32 years old	↓ GM volume in medial temporal, fusiform, and cerebellar regions ↓ WM volume in brainstem and portions of cerebellum
Mean age of 33 years old	↓ GM density in frontostriatal and cerebellar regions, widespread differences in WM
Mean age of 33 years old	↓ density in inferior frontal gyrus pars opercularis, inferior parietal lobule, superior temporal sulcus, middle temporal gyrus, motor cortex, prefrontal cortex, anterior cingulate, medial parietal cortex, supramarginal gyrus, and middle and inferior temporal cortex

Table 8. MRI studies of ASD from childhood to adulthood. WM: white matter, GM: grey matter. Adapted from "Structural MRI in Autism Spectral Disorder", by R. Chen et al., 2012, *Pediatric Research*, 69(5 Pt2): 63R-68R.

3.3.2.1. Gut microbiota

The gut microbiota of children with ASD is characterized by a dysbiosis, even though studies report discordant findings[309]. The preclinical studies on ASD-like models have demonstrated that toxins and bacteria produced by the pathogenic microbiota lead to the activation of immune responses, as seen with increased levels of pro-inflammatory cytokines (e.g. IL-1β, IL-6, Il-8, and IL12p40), and increased gut permeability. Then, cytokines may activate the vagal system which, in turn, regulates the central nervous system activity[310].

3.3.2.2. Cholinergic system

In adults with ASD, a decrease in muscarinic M_1 receptor binding is present in cortical regions while acetylcholine transferase and M2 receptor bindings remain similar to controls[311]. This decrease in adulthood might reflect an earlier change, as the M1 loss in the frontal lobe is also present in children with ASD (data not published[312]). In addition, nicotinic acetylcholine receptors (nAChRs) binding activity in adults is lower in the parietal and frontal cortices, but not in the thalamus nor cerebellum[311,313,314]. nAChRs lower binding activity is associated with changes in receptor subunits. In the parietal cortex, α4 subunit mRNA levels, protein expression, and α4/β2 receptor activity are lower in patients with ASD. In the cerebellum, α4 subunit mRNA levels is elevated, protein expression and α4/β2 receptor activity are low, and α7 subunit binding activity is increased across all cell layers[313,314]. In the paraventricular and reuniens nuclei of the thalamus, α7 and β2 protein expression levels are also reduced[315]. Thus, alterations mainly occur in the nicotinic cholinergic system, but the role of these receptors in the pathology of ASD is not known in humans.

In the BTBR mouse model of autism where decreased levels of acetylcholine and increased levels of kynurenic acid are found in the medial prefrontal cortex, the nicotine cholinergic system modulates social and repetitive behaviors. Indeed, the administration of acetylcholinesterase inhibitors improve social but not the repetitive behaviors in these mice. On the other hand, nicotine administration at low doses improve the social behavior of these mice, while high doses improve their repetitive behavior[316,317]. These studies further support the involvement of the cholinergic system in the pathobiology of ASD, and the role of nAChRs in the modulation of social and repetitive behaviors.

3.3.2.3. Dopaminergic system

In recent years, the dopaminergic system has been increasingly implicated in ASD. In children, mutations of dopaminergic genes encoding dopamine transporter and receptors have been associated with ASD's repetitive and social behaviors[318,319]. ASD children also present a reduction in the release of dopamine in the PFC, and reduced neural response of the mesolimbic reward circuit[320]. In addition, the modulation of the dopaminergic system by administration of risperidone (dopamine receptor D2 antagonist) improves ASD symptoms in children[321]. In adulthood, the dopaminergic system is also altered, as seen with the dopaminergic transporter (DAT) binding levels which are higher in the medial frontal region of the orbitofrontal cortex compared to controls[322].

The mesolimbic pathway, as in humans, is altered in the BTBR mouse model of ASD. Indeed, these mice exhibit blunted functional MRI reactivity in the mesolimbic terminals of the ventral striatum. These changes are associated with a hypofunctionality of pre- and postsynaptic dopamine receptors D2, and reduced adenosine 2a receptor ($A_{2a}R$)-mediated neurotransmission, whereas dopamine receptor D1 (Drd1)-mediated signaling remains similar to controls[323]. Furthermore, in knockout dopamine receptors mouse models, DAT inhibition in substantia nigra neurons results in increased dopamine function in the dorsal striatum and ASD-like behaviors. These dopamine-dependent effects in the striatum are modulated by Drd1, as its blockade leads to the non-appearance of the ASD-like behaviors[324]. Hence, dopaminergic transmission might be altered in a similar way in humans as in mice.

3.3.2.4. Serotoninergic system

In ASD children, serotonin (5-HT) blood, platelet and urine levels are higher than in controls[325,326]. This phenomenon, also called hyperserotonemia, is present in 20 to 30 % of the ASD population[327].

A few studies have evaluated the components of the 5-HT system to test if the 5-HT uptake was different in ASD patients with or without hyperserotonemia. Patients with hyperserotonemia have higher whole blood 5-HT levels and platelet $5HT_2$ receptor binding, but similar platelet 5-HT uptake and thrombin-stimulated release[328,329]. In addition, in adult patients with ASD (hyperserotonemia not referenced) compared to controls, serotonin transporter (SERT) binding levels are lower in the global brain. The most striking reductions are found in the frontal, temporal, parietal, and occipital lobes, as well as in the limbic region, subcortical region, and cerebellum[322]. Altogether, these findings suggest that synaptic 5-HT might also be altered.

However, direct assessment of synaptic 5-HT is not possible in humans, hence the need of animal models to study it.

The SERT Ala56 mouse model of ASD exhibits hyperserotonemia, increased 5-HT clearance of the brain, and increased p38-MAPK-dependent phosphorylation of SERT[330]. The 5-HT brain clearance is associated with enhanced $5HT_{1A}$ and $5HT_{2A}$ receptor sensitivity to agonist drugs. The alteration of 5-HT receptor signaling induces a decrease in the firing rate of the dorsal neurons of the raphe, and bath application of 5-HT increases the inhibition of the neuronal firing of these neurons[330]. The alteration of the 5-HT system during development influence neuronal migration and synapse development, and lead to altered sensory and medial prefrontal cortex development[331,332].

3.3.2.5. Serotonin-N-acetylserotonin-melatonin pathway

In the pineal gland, serotonin is converted by the N-acetyl transferase into N-acetylserotonin (NAS) which, in turn, is converted into melatonin by the Acetylserotonin O-Methyltransferase (ASMT). The principal role of melatonin is to regulate circadian and seasonal rhythms.

In ASD patients, NAS platelet and whole blood serotonin levels are increased while melatonin plasmatic level is lower than for controls[333]. This decrease is associated with mutations in the gene encoding for ASMT, and a decrease in blood ASMT activity, hence limiting the production of melatonin at night[334]. Therefore, the combination of serotonin, NAS, and melatonin levels might serve as a tool to discriminate between ASD patients and controls. However, the mechanisms by which the serotonin-N-acetylserotonin-melatonin pathway influence the pathobiology of ASD remain to be identified.

3.3.2.6. Oxytocinergic system

In children with ASD, contrary to controls, oxytocin (OT) plasmatic levels are low and do not increase with age[335], and unprocessed OT peptide forms are increased[336], suggesting that low levels of OT are associated with ASD. Moreover, oxytocin receptors (OTR) single-nucleotide polymorphisms have also been associated with ASD[337]. Treatment with intranasal or intravenous OT administration in ASD patients is associated with enhanced social behaviors[338,339], further promoting the role of the oxytocinergic system in the physiopathology of ASD. However, little is known about the mechanisms underlying these effects.

Studies of OT and OTR in animal models have also supported the role for OT in social behavior. Indeed, OT and OTR knockout mice display social deficits such as impaired social memory

and cognitive flexibility[340]. In voles, social deprivation and enrichment modulate the synthesis of OT and OTR binding[341]. Furthermore, blockade of OT by its antagonist in pregnant dams results in offspring exhibiting autistic-like behaviors[127]. Therefore, early alterations in the oxytocinergic signaling induce long-term social deficits. Nonetheless, the environment also modulates the oxytocinergic pathway, even though the mechanisms of action are not known.

3.3.2.7. Glutamatergic system

Children with ASD have a higher glutamate plasma level than controls. This may be correlated to: (1) high brain glutamate levels[342], (2) lower glutamine levels[343], and (3) increase in the glutamate/glutamine ratio[343]. In addition, in children and adults with ASD, a selective reduction in glutamic acid decarboxylase (GAD; enzyme converting glutamate into GABA) mRNA is found in dentate nuclei cells and might result in increased glutamate levels in the brain[344].

The changes in glutamine and glutamate levels might be linked to an increase in gliosis activity which can disturb enzymatic regulation and may therefore indirectly alter the metabolism of glutamate and glutamine[345]. Also, decreased NMDA glutamate receptor function is associated with reduced social interaction, social communication, and repetitive behaviors in mice[346]. Moreover, in the fragile X and valproate rodent models of ASD, increased spontaneous glutamatergic activity is observed at birth and 15-day old offspring (P15). Therefore, the glutamatergic system is also involved in the pathophysiology of ASD[127].

3.3.2.8. GABAergic system

Over the past years, the GABAergic system has been extensively studied in the pathophysiology of ASD in humans. Modifications of this system are summarized in Table 9.

Moreover, the excitation/inhibition imbalance hypothesis suggests that a GABAergic deficit is present in the pathophysiology of ASD, hence the need of animal studies to test it. Numerous animal models of autism have been used to further understand the role of the GABAergic system in the pathophysiology of ASD, as summarized in Table 10.

	Age of patients	Findings relative to control
In vivo studies	4-12 years old[347,348]	↑ GABA plasma levels
	Mean of 5.2 years old[349]	↓ GABA levels in left frontal lobe, no change in lenticular nuclei
	Mean of 7 years old[350]	↑ GABA plasma levels
	Mean of 7.3 years old[349]	↓ $GABA_AR$ levels in superior and medial frontal cortex
	Mean of 7.8 years old[351]	↑ GABA plasma levels
	Mean of 8 years old[343]	↑ GABA blood levels
	Mean of 8 years old[352]	↑ GABA plasma levels associated with ↑ hyperactivity, impulsive severity, tip toeing severity, light sensitivity, and tactile sensitivity
	Mean of 9.9 years old[353]	↓ GABA platelet levels
	Mean of 14.01 years old[354]	↓ GABA levels in left perisylvian region
	Mean of 29.61 years old[355]	↓ GABA action on perceptual suppression in the visual system
	Mean of 39.3 years old[356]	↓ α5 $GABA_AR$ subunit in Nacc and subcallosal area
Studies from autopsy brain tissues	13-54 years old[357]	↑ interneuronal packing density of parvalbumin-positive interneurons in CA1, CA3, and anterior body of the hippocampal formation, ↑ calbindin neuronal density in dentate gyrus, ↑ calretinin neuronal density in CA1
	14-30 years old[358]	↓ GABBR binding density in superficial layers of anterior cingulate cortex, all layers of posterior cingulate cortex and fusiform gyrus
	16-24 years old[359]	↓ $GABA_AR$ binding in CA1 stratum pyramidale
	16-30 years old[360]	↓ GAD67 mRNA levels in Purkinje cells
	16-30 years old[344]	↓ GAD65 mRNA levels in dentate nucleus
	19-22 years old[361]	↓ $GABA_AR$ levels in whole hippocampus
	19-28 years old[362]	Cerebellum: ↑ GABRα2, GABRα3, GABRβ3, GABRɣ2, GABRɣ3, GABRθ and ↓ GABRα6, GABRβ2 mRNA levels BA40: ↑ GABRɣ3 and ↓ GABRα3 mRNA levels BA9: ↑ GABRα6 and ↓ GABRα2, GABRα3, GABRβ3, GABRɣ3, GABRθ mRNA levels, ↓ GABRα6, GABRβ2, GABRδ, GABRε, GABRɣ2, GABRρ2 protein levels
	19-30 years old[363]	↓ GAD65 protein levels in cerebellum, ↓ GAD67 protein levels in parietal cortex
	19-30 years old[364]	↓ GABBR1 expression in cerebellum, BA9 and BA40, ↓ GABBR2 expression in cerebellum
	19-30 years old[365]	Cerebellum: ↓ GABRα1, GABRβ3 protein levels BA40: ↓ GABRα1, GABRα2, GABRα3, GABRβ3 protein levels BA9: ↓ GABRα1 protein levels
	19-30 years old[366]	↓ $GABA_AR$ binding in supra-and infragranular layers of the anterior cingulate cortex
	19-30 years old[367]	Cerebellum: ↓ GABBR1 protein levels, ↑ GABRα4, GABARα5, GABRβ1, GABBR1 mRNA levels BA40: ↓ GABRα5 and GABBR1 protein levels, ↑ GABBR1 mRNA levels BA9: ↓ GABRα4, GABRα5, GABRβ1, GABBR1 protein levels, ↓ GABRα4, GABRα5, GABRβ1 mRNA levels

Table 9. Summary of changes in the GABAergic system of patients with ASD. BA9: Brodmann area 9, BA40: Brodmann area 40, GABBR: $GABA_B$ receptor, GABR: $GABA_A$ receptor, Nacc: nucleus accumbens.

Animal models	Structure	Age	Alterations in GABAergic signaling
MECP2$^{-/-}$ mice	Brainstem[368]	P7	Depression in GABAergic transmission in the ventrolateral medulla
	Cx and striatum[369]	Adult	↓ mRNA levels of GAD65 and GAD67
	HPC[370]	Adult	Depression in GABAergic transmission, ↓ frequency of IPSC-based spontaneous rhythmic field potentials
	LC[371]	P14-P28	Depression in GABAergic transmission in norepinephrinergic neurons
	Primary SM cx[369,372]	P14-P35 Adult	E/I balance shifted to favor inhibition in L5 pyramidal neurons ↓ inhibitory quantal size in L2/3 of pyramidal neurons
FMR1$^{-/-}$ mice	BL-AM[373]	P20-P30	↓ in amplitude and frequency of sIPSCs and mIPSCs in pyramidal cells, ↓ GAD65/67 levels
	Cortex[374]	Adult	↓ mRNA levels of $GABA_AR$ α1, α3, α4, β1, β2, δ, ɣ1, ɣ2 subunits
	Forebrain[375]	P5	↓ expression of $GABA_AR$ α1 subunit
		P12	↓ expression of $GABA_AR$ α1, β2, δ subunits, ↓ expression of succinic semialdehyde dehydrogenase and GABA-T
		Adult	↓ expression of $GABA_AR$ β2 subunit, ↑ expression of GAD65
	HPC[127,376]	E20-P30	Persistent depolarizing effect of GABA in pyramidal cells Abolition of the oxytocin-mediated hyperpolarizing shift at birth
		Adult	↓ $GABA_AR$ subunit δ expression
	Neocortex[376,377]	P14-P30	E/I balance shifted to favor excitation over inhibition
		Adult	↓ $GABA_AR$ subunit δ expression
	SM Cx[378,379]	P5-P15	Delayed switch of GABA polarity in spiny stellate neurons of L4
		Adult	↓ number of PV-positive interneurons in neocortical inhibitory circuit
	Striatum[380]	Adult	↑ frequency of sIPSCs and mIPSCs
	Subiculum[381]	Adult	Down regulation of $GABA_A$-mediated tonic inhibition, ↓ expression of α5 and δ GABAAR subunits
	WB[382]	Adult	↓ expression of $GABA_AR$ β subunit in Cx, HPC, brainstem and diencephalon, ↓ expression of GAD in Cx, HPC, brainstem and diencephalon
GABRB3$^{-/-}$ mice	Parietal cx[383]	Adult	E/I balance is shifted to favor excitation over inhibition
	WB[384]	Adult	↓ $GABA_AR$ expression, particularly in HPC and cerebral cortex
RELN$^{-/-}$ mice	WB[385]	Adult	↓ levels of GAD67, ↓ GABA turnover
NL3^{R451C} KI mice	Barrel cx[386]	P9-P15	↓ GABA signaling between PV basket cells and spiny neurons in L4
	HPC - CA1[387]	P21-P35	↓ GABAergic synaptic transmission at synapses formed by PV basket cells onto pyramidal cells, ↑ GABAergic synaptic transmission at pyramidal synapses formed by CCK basket cells
	HPC - CA3[388]	P4-P35	↑ frequency of GDPs and mIPSCs
	Neocortex[389]	P21-P35	Asymmetric ↓ of PV positive cells
	SM cx[390]	P13-P16	↑ frequency of mIPSCs in L2/3, ↑ VGAT and gephyrin levels
Valproic acid rodents	Cortex[391]	P23-P45	↓ $GABA_A$-mediated neurotransmission in L2/3 cells
	HPC[127]	E20-P30	Persistent depolarizing effect of GABA in pyramidal cells Abolition of the oxytocin-mediated hyperpolarizing shift at birth
	Lateral AM[392]	P12-P16	E/I balance shifted to favor inhibition in pyramidal neurons
	Neocortex[389]	P21-P35	Asymmetric ↓ number of PV positive cells
UBE3A-deficient mice	Cerebellar cortex[393]	P25-P28	↓ $GABA_A$-mediated tonic inhibition
Scn1a$^{+/-}$ mice	HPC - CA1[394]	P21-P30	E/I balance shifted to favor excitation over inhibition in interneurons
En2$^{-/-}$ mice	HPC and SM cx[395]	Adult	↓ number of PV and SOM interneurons in hilus, ↓ number of PV and SOM interneurons in L2/3

Table 10. Alterations of GABAergic signals in animal models of ASD. AM: amygdala, BL-AM: basolateral nucleus of the amygdala, CCK: cholecystokinin, Cx: cortex, HPC; hippocampus, LC: locus coeruleus, NPY: neuropeptide Y, PV: parvalbumin, SM cx: somatosensory cortex, SOM: somatostatin, VGAT: vesicular GABA transporter, WB: whole brain. Adapted from "GABAergic signaling as therapeutic target for autism spectrum disorders", by G. Cellot and E. Cherubini et al., 2014, *Frontiers in Pediatrics*, 2(July): 1-11.

During my thesis work, I focused on the GABAergic system and its relationship with ASD by relying on previous studies done in our lab to test if C-section delivery might lead to ASD. Indeed, our lab has shown that the oxytocin-mediated GABA hyperpolarizing shift at birth is altered in the hippocampus (CA3) of two rodent models of autism. Also, the GABA developmental shift from excitation to inhibition (E to I) during the second postnatal week is abolished, hence a persistent depolarizing GABA effect in hippocampal CA3 pyramidal cells is present in ASD rodent offspring. The restoration of the transient hyperpolarizing GABA shift at birth by the administration of bumetanide (NKCC1 antagonist) leads to the restoration of the postnatal GABA switch from E to I[127] in CA3 pyramidal cells.

Based on these observations, we hypothesized that a disruption of the mode and time of delivery might lead to an alteration of the GABA developmental sequence in CA3 pyramidal cells and induce a persistent depolarizing effect in those cells such as the one observed in two models of autism.

4. HIPPOCAMPUS

The hippocampal formation, originating from the medial pallium of the telencephalon, includes the dentate gyrus (DG) and *cornu ammonis* (CA) which constitute the hippocampus *per se*, the subiculum, presubiculum, parasubiculum, and entorhinal cortex (EC). The DG is composed of the fascia dentata and the hilus, and the CA is subdivided in 3 regions, CA1, CA2 and CA3 which are laminated in characteristic strata (Figure 7). This structure, together with the cingulate cortex, olfactory cortex, and amygdala, belongs to the limbic system and is functionally associated with spatial learning, and consolidation and retrieval of episodic memory.

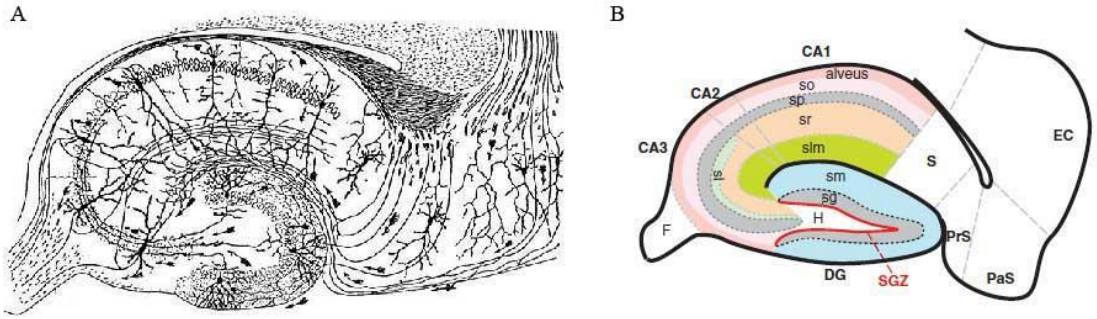


Figure 7. Description of the hippocampal formation. (A) Picture of the hippocampus by Santiago Ramon y Cajal in 1911. (B) Schematic layer organization in the mature hippocampus. So: stratum oriens, sp: stratum pyramidale, sr: stratum radiatum, slm: stratum lacunosum moleculare, sm: stratum moleculare, sl: stratum lucidum, sg: stratum granulosum, F: fimbria, S: subiculum, PrS: presubiculum, PaS: parasubiculum, EC: entorhinal cortex, H: hilus, DG: dentate gyrus, SGZ: subgranular zone. Reprinted from "The development of hippocampal cellular assemblies", by G. Li and S.J. Pleasure, 2014, *Wiley interdisciplinary reviews. Developmental biology*, 3(2): 165-177.

4.1. Neurogenesis

The hippocampus contains numerous types of neurons which have specific genesis pathways and can be divided into three main categories: pyramidal cells, interneurons, and granular cells.

4.1.1. Pyramidal cells

Pyramidal cells from CA1 and CA3 are primarily generated in the ventricular (VZ) and subventricular (SVZ) zones (Figure 8). CA1 neurons are generated in rats between embryonic day 16 (E16) and E20 with a peak around E18-E19, and in mice between E12 and E18 with a peak around E14-E16[396–399]. CA3 neurons are generated slightly earlier; in rats between E16 and E20 with a peak around E17-E18, and in mice between E11 and E18 with a peak around E13-E15[396–399]. For CA2 neurons which are generated in mice between E10 and E15 with a peak at E13, their migration will not be further described as little is known about it[399].

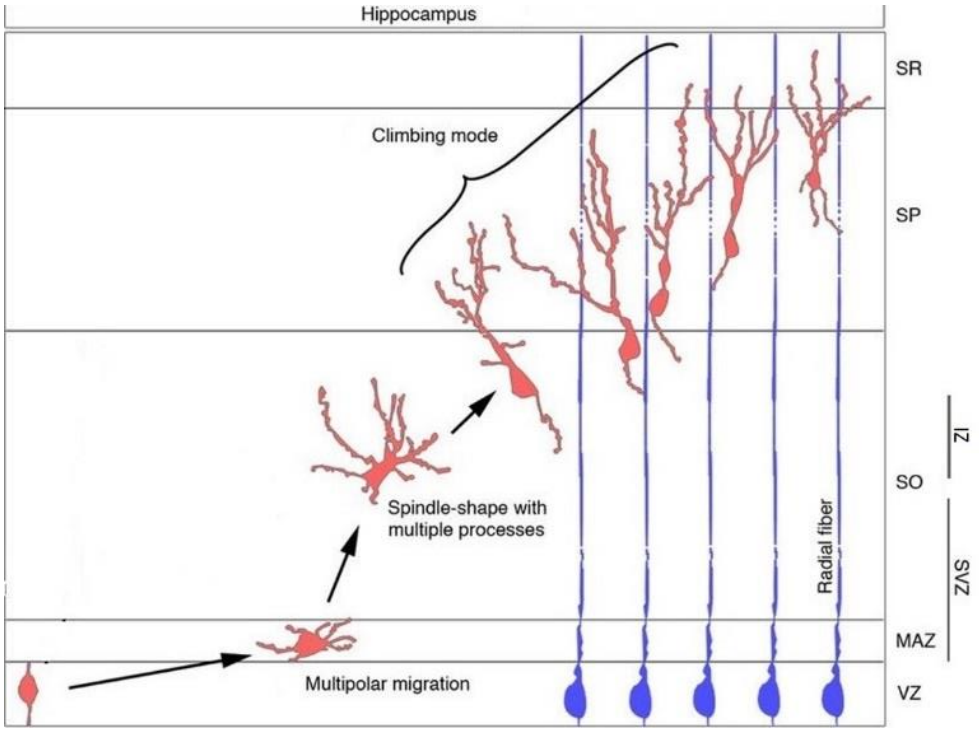


Figure 8. Schematic representation of the migratory behavior of hippocampal cells. VZ: ventricular zone, SVZ: subventricular zone, MAZ: multipolar cell accumulation zone, SO: stratum oriens, SP: stratum pyramidale, SR: stratum radiatum. Adapted from "Hippocampal pyramidal neurons switch from a multipolar migration mode to a novel "climbing" migration mode during development", by A. Kitazawa et al., 2014, *The Journal of Neuroscience*, 34(4): 1115-1126.

CA1 and CA3 early-born pyramidal neurons, in parallel to their migration in the multipolar cell accumulation zone (MAZ), transform into multipolar cells and then, further differentiate into bipolar spindle-shaped cells and migrate toward the intermediated zone. (IZ).

In CA1, multipolar cells differentiation time depends on their date of birth, as neurons generated at E12 or E13 remain multipolar for 1 day, whereas neurons generated at E15-16 accumulate in the MAZ as multipolar cells for 3-4 days[400]. Spindle-shaped neurons migrate along with radial glial fibers toward the future stratum pyramidale, also called hippocampal plate (HP)[401]. Upon their entrance in the HP, spindle-shaped neurons extend branched leading process(es) to contact radial glial fibers. In addition, the soma of these cells moves along every newly formed leading process in a "climbing mode" until they reach the top of the HP[400].

CA3 pyramidal neurogenesis is neurogenic gradient-dependent, thus cells near CA1 are generated earlier than those closer to the DG. In addition, CA3 multipolar cells are hypothesized to sojourn for a longer time in the MAZ than CA1 neurons because they must wait for a connection with DG cells[397]. The later migration and differentiation of CA3 cells is similar to the ones for CA1 cells, even though multiple migration modes may exist depending on the birth date of CA3 neurons[396].

Differentiation between CA1 and CA3 cells is thought to be determined soon after the multipolar stage, as CA1 and CA3 neurons in mice already express differential specific markers in the IZ (around E15.5)[402].

4.1.2. Interneurons

Interneurons originate from the medial ganglionic eminence (MGE) and the caudal ganglionic eminence (CGE) of the subpallium telencephalon and migrate tangentially to the cortex towards the hippocampus. Interneurons generated from the MGE migrate toward CA regions, while interneurons from the CGE migrate toward both CA regions and DG[403].

MGE early-born neurons migrate within the IZ, while late-born neurons migrate within the SVZ. Interneurons later migrate radially into the cortical plate for early-born neurons, and in the marginal zone (MZ) for late-born neurons. Interneurons further differentiate and spread via tangential migration, ventricle directed migration, and radial migration before reaching their final position in the cortex and CA regions. CGE neurons migrate caudally toward the MZ and later to CA regions and DG.

The hippocampal formation is colonized by interneurons via two pathways, a superficial one (major stream) in the MZ, and a deep one in the SVZ/lower IZ (deep stream). The major stream reaches the subiculum and CA1 field at E15, CA3 at E16, and the dentate gyrus primordium at E17. The deep stream stops at the border between the neocortex and subiculum at E15, reaches CA1 at E16, and the dentate gyrus primordium at E17[404].

4.1.3. Granular cells

The granular cells of the DG are primarily generated in the primary dentate neuroepithelium located around the dentate notch at E16-E17 in rats[405] and E13.5-E14 in mice[406]. Newborn granule cells migrate to the secondary dentate matrix, and later to the subpial region. The first granular cells reaching the subpial region differentiate into unipolar and bipolar cells and migrate along the radial glial fibers to the outer shell of the supra-granular blade of the DG. Granular cells generated later migrate to form the outer shell of the infra-granular of the DG. This migration is called “dentate migration” and is over around E17.5-E18.5 in mice[406].

The “second dentate migration” that takes place during late embryonic and early postnatal days consists in granular cells migrating to form the tertiary dentate matrix (TDM). Neurons generated from the TDM later form the inner part of the DG. Therefore, early-born granular

cells form the outer part of the DG, while late-born neurons form its inner part. Moreover, this developmental pattern seems to be conserved across species[405,407].

The DG has also been shown to continuously generate new neurons during adulthood, even though this finding remains debated at the moment[408]. One possible explanation is that adult and early postnatal neurogenesis seem to use a common unified process, yet immature granule cells are only detected in young animals[409].

4.2. Neuronal populations

4.2.1. Pyramidal and granular cells

Pyramidal and granular cells account for approximately 80-90% of the hippocampal neurons. These glutamatergic cells are responsible for the excitatory neurotransmission in the hippocampus.

The somas of granular cells are grouped into the dense layer *stratum granulosum* of the DG, while somas of pyramidal cells are mostly grouped into the compact layer *stratum pyramidale* of the CA. Excitatory inputs on these cells terminate exclusively on dendritic spines of distal *strata radiatum* and *oriens*, while inhibitory inputs target dendritic shafts of proximal dendritic segments of *strata radiatum* and *oriens* (~70%), soma, and axon initial segment[410].

Even though pyramidal cells are generally presented as a single population, they can actually be divided into 3 subpopulations: (1) cells with soma in *stratum pyramidale* close to *stratum radiatum* and weakly calbindin-positive (a Ca^{2+} binding protein), (2) cells with soma in *stratum pyramidale* close to *stratum oriens* and calbindin-negative, and (3) cells located in *stratum radiatum* and previously called radiatum giant cells[411].

4.2.2. Interneurons

Interneurons are GABAergic cells responsible for the inhibitory activity in the adult hippocampus and regulate the timing of pyramidal cell activity. Inhibitory inputs on these cells are mostly present in the perisomatic region, while excitatory inputs are distributed across the whole cell[412]. Contrary to pyramidal cells, interneurons are a heterogenous population with distinct properties and specific markers after maturation during early postnatal life. They can be divided into different subpopulations either by their specific marker including Ca^{2+}-binding proteins, or by their axonal arborization (Figure 9)[403].

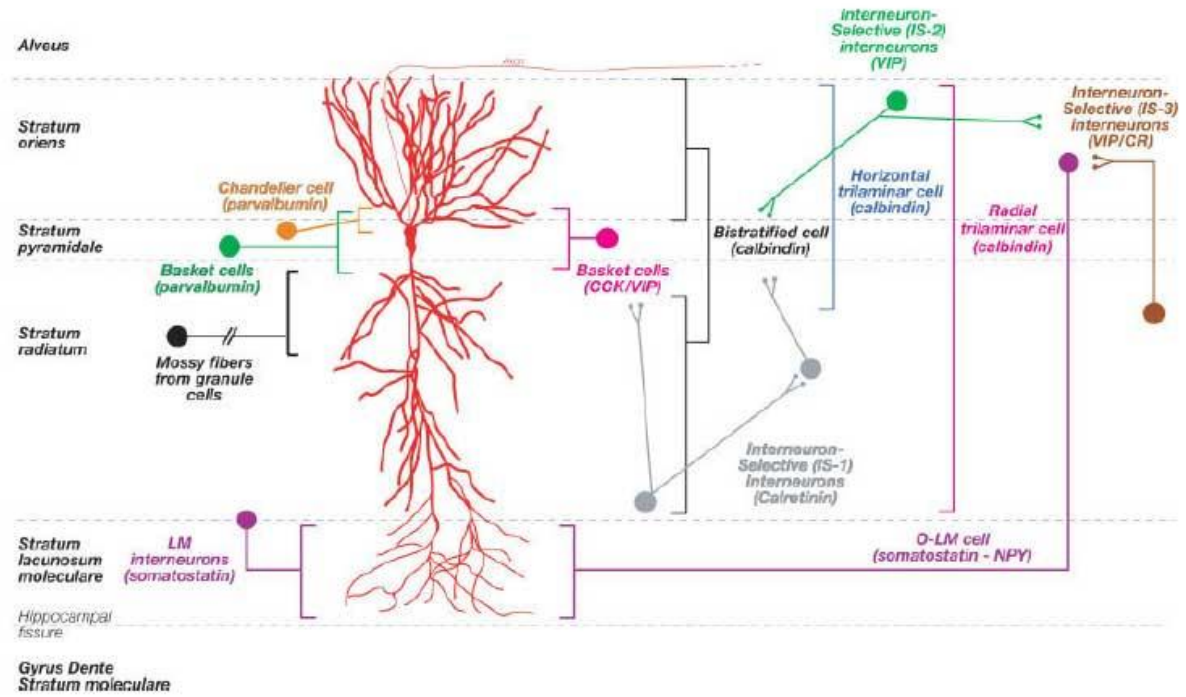


Figure 9. Schematic representation of the GABAergic afferences on hippocampal pyramidal cells. CCK: cholecystokinine, CR: calretinin, IS: interneuron-selective, LM: lacunosum-moleculare, NPY: neuropeptide Y, O-LM: oriens-lacunosum-moleculare, VIP: vasoactive intestinal polypeptide. Reprinted from "The Development of Hippocampal Interneurons in Rodents" by L. Danglot et al., 2006, *Hippocampus*, 16(2006): 1032-1060.

Interneurons located in the *stratum pyramidale* are chandelier cells, basket cells, bistratified cells, and radial trilaminar cells. Basket cells containing either parvalbumin (PV) or cholecystokinin/vasoactive intestinal peptide (CCK/VIP) innervate the perisomatic region of pyramidal cells, while chandelier cells containing PV innervate the initial segment of pyramidal cells. Bistratified cells express calbindin and innervate the dendrites of pyramidal cells. Radial trilaminar cells also express calbindin and innervate the pyramidal cell except for the distal part of dendrites located in the *stratum lacunosum moleculare*[403].

Lacunosum-moleculare interneurons (LM) are located at the border between *stratum radiatum* and *stratum lacunosum moleculare*. LM cells expressing somatostatin innervate the distal portion of pyramidal cell dendrites in the *stratum lacunosum moleculare*[403].

Interneurons present in the *stratum radiatum* are interneuron-selective interneurons (IS): IS-1 expressing calretinin and IS-3 expressing VIP/calretinin. IS-1 innervate basket cells expressing CCK/VIP and other IS-1 cells, whereas IS-3 interneurons innervate oriens-lacunosum-moleculare (O-LM) cells[403].

Interneurons located in the *stratum oriens* are IS-2 expressing VIP and O-LM interneurons expressing somatostatin and neuropeptide Y (NPY). IS-2 innervate interneurons contacting

pyramidal cells and IS-3 cells. O-LM cells, similar to LM cells, innervate the distal portion of pyramidal cell dendrites in the *stratum lacunosum moleculare*[403].

4.3. Synaptic transmission

The tri-synaptic loop enabling the unidirectional synaptic transmission between hippocampal regions was first described by Santiago Ramon y Cajal (Figure 7A). This circuit starts with axons from layer 2 of the EC projecting to the DG and CA3 through the perforant pathway[413] (Figure 10). Granule cells in the DG project through their mossy fibers (unmyelinated axons) to the proximal dendrites of CA3 pyramidal cells, and to mossy cells and interneurons in the hilus[414]. Then, CA3 pyramidal cells project to CA1 and CA3 though Schaffer collaterals[415] (Figure 10). CA1 pyramidal cells project to the subiculum and EC deep layers, which, in turn, projects back to many cortical areas including the ones that originally projected into the hippocampus[416]. Hence, sensory information coming from the EC is processed in the hippocampus and transmitted back to the cortical regions of origin (Figure 10).

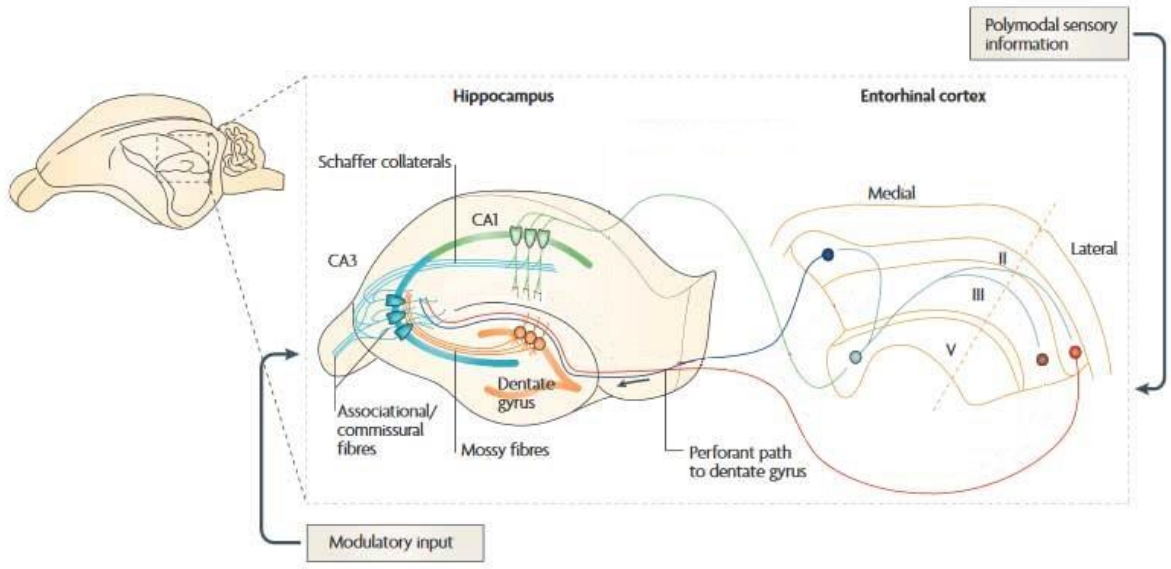


Figure 10. Tri-synaptic loop of the hippocampal formation. Adapted from "Synaptic plasticity, memory and the hippocampus: a neural network approach to causality", by G. Nerves et al., 2008, *Nature Reviews Neuroscience*, 1(9): 65-75.

The role of CA2 in this tri-synaptic loop has been ignored for a long time. It was first assumed that CA2 pyramidal cells form a minor pathway linking CA3 to CA1[417]. However, more recent studies found that CA2 neurons mediate a disynaptic pathway in this cortico-hippocampal loop[418,419]. EC neurons from layers 2/3 send strong excitatory inputs to CA2 neurons which, in turn, project excitatory inputs to CA1 pyramidal neurons. On the other side, inputs from CA3 pyramidal neurons strongly inhibit CA2 neurons[419] (Figure 11).

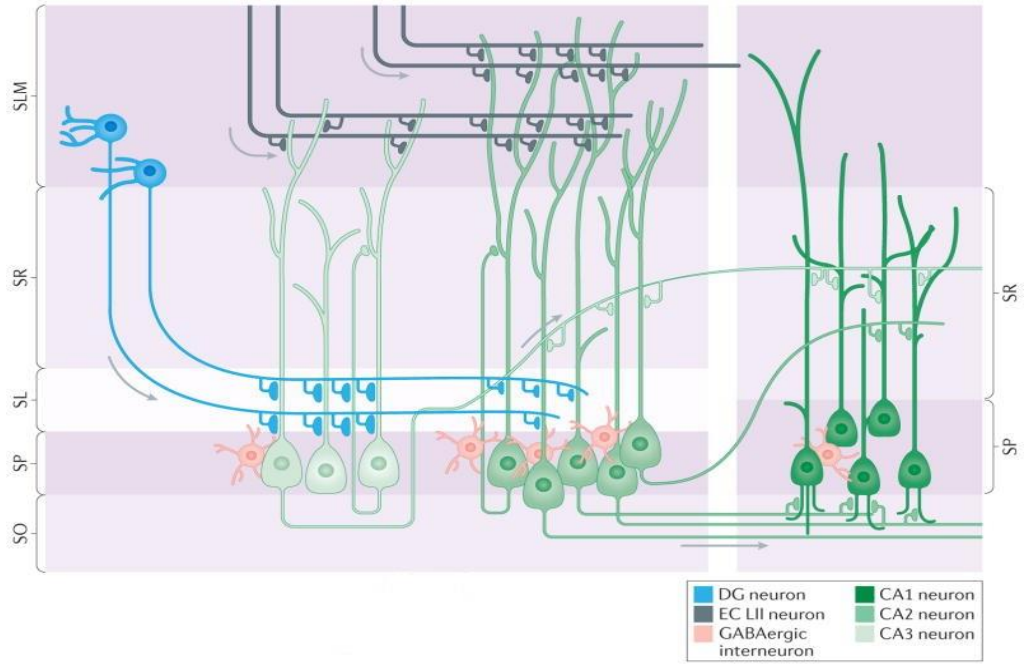


Figure 11. Di-synaptic loop of the hippocampal formation. SLM: stratum lacunosum-moleculare, SR: stratum radiatum, SL: stratum lucidum, SP: stratum pyramidale, SO: stratum oriens. Adapted from "Rediscovering area CA2: unique properties and functions", by S.M. Dudek et al., 2016, *Nature Reviews Neuroscience*, 17(2): 89-102.

Therefore, synaptic transmission in the hippocampus is mediated through 2 pathways: the tri-synaptic loop (EC layer 2 – DG – CA3 – CA1) and the di-synaptic pathway (EC layer 2/3 – CA2 – CA1).

4.3.1. Glutamatergic neurotransmission

The main excitatory neurotransmitter in the central nervous system (CNS) is glutamate. Its actions are mediated by two classes of glutamate receptors: ionotropic and metabotropic receptors.

4.3.1.1. Ionotropic receptors

Ionotropic receptors are ligand-gated ion channels subdivided into three categories and named due to their selective agonist: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-aspartate), and kainate receptors. These receptors are composed of four subunits forming a heteromeric structure permeable to Na^+ and K^+ ions. NMDA and specific isoforms of kainate and AMPA receptors are also permeable to Ca^{2+} ions.

AMPA receptors are formed by the combination of four subunits: GluA1, GluA2, GluA3, and GluA4, each combination conferring different properties to the receptor[420]. For example, GluA2-lacking AMPA receptors have higher conductance and are Ca^{2+}-permeable. In addition, the post-transcriptional RNA editing at the Q/R site of the GluA2 subunit determines important

properties for AMPA receptors. Indeed, the replacement of glutamine (neutrally charged) by arginine (positively charged) at the Q/R site results in low conductance, Ca^{2+}-impermeable receptors[421].

NMDA receptors are multisubunit channels composed of subunits GluN1, GluN2A-D, and GluN3A-B. These receptors are voltage-gated channels whose opening requires the presence of glutamate and co-agonist glycine, as well as the removal of the Mg^{2+} blockade, to open. The Mg^{2+} block is removed by a depolarization of sufficient amplitude and duration of the cell membrane[422]. NMDA receptors have slower kinetics compared to AMPA and kainate receptors, and their activation results in the entrance of Ca^{2+} required for synaptic plasticity mechanisms[423].

Kainate receptors are heteromers composed of subunits GluK1, GluK2, GluK3, GluK4, and GluK5. Subunits GluK1-3 can form homo- or heteromers, where GluK4 and Gluk5 can only form heteromers with subunits GluK1-3. Kainate receptors are expressed throughout the nervous system and have pre- and postsynaptic roles. Activation of these receptors generate slow and low amplitude postsynaptic responses[424], and modulate neuronal excitability[425].

4.3.1.2. Metabotropic receptors (mGluRs)

Glutamate metabotropic receptors induce slow postsynaptic responses that modulate postsynaptic cells excitability, inhibit presynaptic release of glutamate and GABA, and participate in synaptic plasticity[426]. mGluRs are G protein-coupled receptors, with eight mGluRs subtypes identified. These receptors are further classified into three groups based on sequence homology, G-protein coupling, and ligand-selectivity: group I, group II, and group III[426].

Group I include mGluR1 and mGluR5 that are located predominantly postsynaptically and coupled to Gαq proteins. Activation of the Gαq protein stimulates phospholipase C (PLC) which, in turn, stimulates adenylate cyclase (AC), leading to the phosphorylation of MAPK. Group II include mGluR2 and mGluR3 that are located both presynaptically and postsynaptically, and group III include mGluR4, mGluR6, mGluR7, and mGluR8 that are located predominantly presynaptically. Group II and group III are both coupled to Gαi proteins whose activation inhibits PLC[426].

4.3.2. GABAergic neurotransmission

Numerous physiological and cognitive processes depend on the "fine-tuning" between excitatory and inhibitory systems. In the adult mammalian brain, GABA is the main inhibitory neurotransmitter regulating synaptic activity, preventing neuronal hyperexcitation as well as oscillatory activity and firing rate impairments in neural networks[427,428].

4.3.2.1. GABA receptors

GABA is synthetized in the cytoplasm of interneurons by the decarboxylation of glutamate by the GAD_{65} and/or GAD_{67} enzymes. GABA actions are mediated by two classes of GABA receptors: metabotropic $GABA_B$ and ionotropic $GABA_A$ and $GABA_C$ (not described here as they have been characterized most extensively in the retina).

$GABA_B$ receptors ($GABA_BR$) are GTP-dependent G-protein coupled metabotropic receptors mediating slow GABA responses. Two heteromeric forms consisting of $GABA_{B1(a\ or\ b)}$ subunits combined with $GABA_{B2}$ subunits. $GABA_BRs$ are linked to G_i/G_o whose activation inhibits AC and modulate voltage-gated Ca^{2+} and protein-gated inwardly rectifying K^+ channels (GIRKs). $GABA_{B1a}$ subunit assembles presynaptically with $GABA_{B2}$, while $GABA_{B1b}/GABA_{B2}$ form predominantly postsynaptically. Presynaptic activation of $GABA_BRs$ inhibits voltage-activated Ca^{2+} channels (N or P/Q types), leading to decreased neurotransmitter release. Postsynaptic activation induces the activation of GIRKs and leads to inwardly rectifying K^+ current, mediating postsynaptic inhibition[429].

$GABA_A$ receptors ($GABA_AR$) are ionotropic pentameric assemblies of distinct subunits and are permeable to chloride (Cl^-) and bicarbonate (HCO_3^-) ions. In the mammalian CNS, 19 subunits have been identified and divided into subfamilies: α1-6, β1-3, ɣ1-3, δ, ε, π, θ, and ρ1-3. Subunits consist of an extracellular N-terminal domain followed by four transmembrane sequences (M1-M4), and a large intracellular loop between M3 and M4 involved in modulation by phosphorylation. Alternative splicing and RNA editing of these subunits contribute to receptor diversity, and functional properties depend both on subunits composition and arrangement[430]. $GABA_ARs$ are generally constituted of 2α, 2β, and 1ɣ or 1δ. $GABA_ARs$ with α1-3, β, and ɣ are located synaptically and binding of GABA to this receptor induces channel opening, thus creating an anion flux (phasic current) and leading to the hyperpolarization of the cell membrane[431]. $GABA_ARs$ α(4/6)βδ are predominantly localized at extrasynaptic sites and induce tonic inhibition, with basal ambient GABA concentrations resulting in the opening of these

receptors for longer periods of time than the previously described receptors[431]. $GABA_AR$ are also present presynaptically on axons of pyramidal and granular cells[431].

4.3.2.2. GABA during development

As mentioned before, GABA is the main inhibitory neurotransmitter in the adult CNS; however, its actions during the neonatal period differ from those in adulthood. Indeed, at early stages, GABA plays multiple roles including (1) depolarization and excitation of immature neurons via $GABA_ARs$, (2) modulates neuronal activity by generating synchronized $GABA_A$-mediated activities, and (3) increases intracellular calcium concentrations, resulting in the activation of numerous cellular pathway including neuronal maturation through brain-derived neurotrophic factor (BDNF) release[432].

4.3.2.2.1. Depolarizing actions of GABA

Early depolarizing actions of GABA have been identified in various brain structures and species by using intracellular, gramicidin-perforated, and cell-attached recordings. Specifically, during fetal and early postnatal life in rodents, GABA is strongly depolarizing and excitatory, with a transient hyperpolarizing and inhibitory shift occurring at birth[127]. In addition, GABA activity at birth is mediated by oxytocin since application of its antagonist right before birth abolished the transient hyperpolarizing shift[127] (Figure 12A). A second and persistent GABA shift from excitation to inhibition occurs during the second postnatal week in rodents. These developmental changes in GABA signaling are associated to a progressive negative shift of $GABA_AR$ reversal potential E_{GABA} (Figure 12B) which, in turn, reflects changes in chloride homeostasis[126]. In immature neurons, binding of GABA to $GABA_AR$ induces a chloride efflux leading to depolarization of the cell membrane. During neuronal maturation, intracellular chloride concentration ($[Cl^-]_i$) decreases and leads to chloride influx after $GABA_AR$ activation and a hyperpolarizing effect[433].

This developmental change in $[Cl^-]_i$ depends on the pivotal role of Na^+-K^+-$2Cl^-$ (NKCC1) and K^+-$2Cl^-$ (KCC2) co-transporters in chloride homeostasis in the CNS. NKCC1 is the principal membrane transporter allowing chloride accumulation inside neurons, whereas KCC2 is the principal transporter allowing its extrusion[429]. Prenatally, NKCC1 expression is high, while KCC2 expression is low, resulting in elevated $[Cl^-]_i$ and GABA excitatory activity. Along with neuronal maturation, NKCC1 expression decreases while KCC2 expression increases, leading to chloride extrusion from neurons and GABA inhibitory activity (Figure 13)[429].

This early GABA excitation is critical for morphological maturation and generation of primitive oscillations.

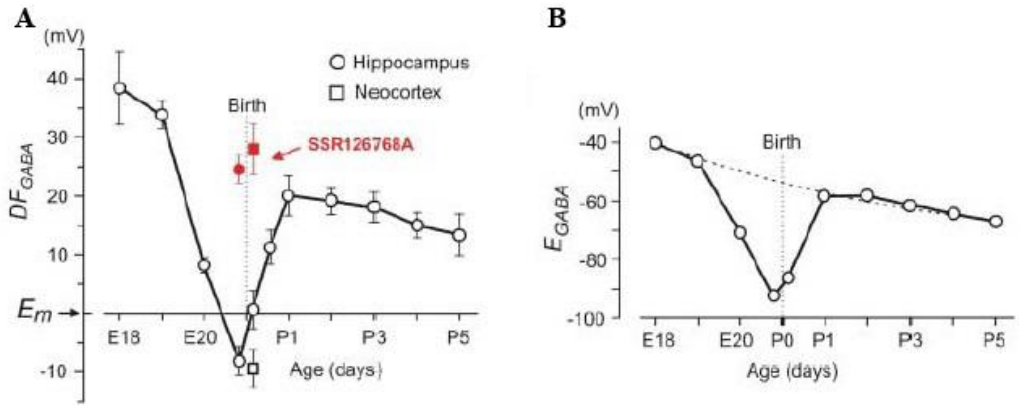


Figure 12. Developmental excitatory to inhibitory GABA sequence. (A) Age-dependence of DF_{GABA} in rats. Red indicates pretreatment with SSR126768A (OTR antagonist). (B) Age-dependence of the $GABA_A$ reversal potential ($E_{GABA}=E_m+DF_{GABA}$) in rats. (○) CA3 pyramidal cells, and (□) neocortical pyramidal cells. Adapted from "Maternal oxytocin triggers a transient inhibitory switch in GABA signaling in the fetal brain during delivery", by R. Tyzio et al., 2006, *Science*, 314(5806): 1788-1792.

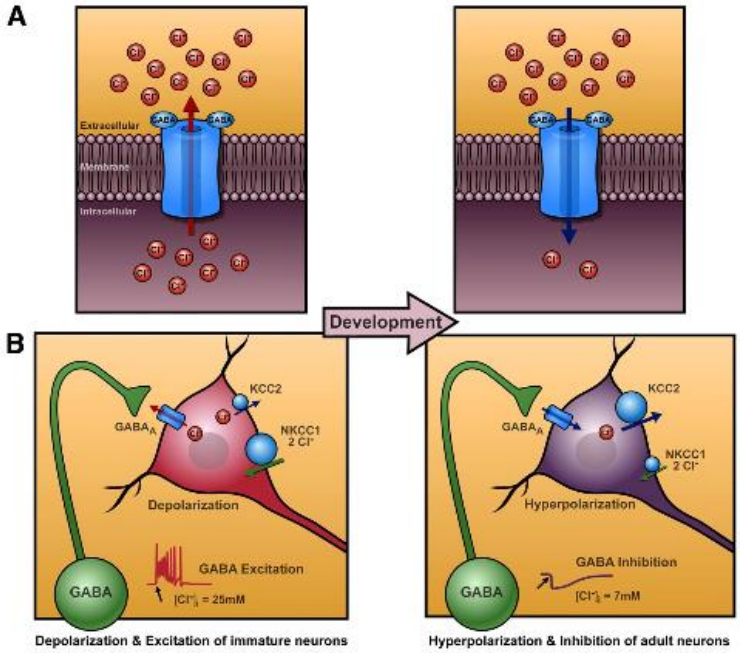


Figure 13. Changes in chloride homeostasis during development. (A) During development, the intracellular chloride concentration decreases. In immature neurons, efflux of the negatively charged chloride ions produces inward electric currents and depolarization. In mature neurons, chloride enters the cell and produces outward electric currents and hyperpolarization. (B) Developmental change in intracellular chloride is due to changes in the expression of the two major chloride cotransporters, KCC2 and NKCC1. Chloride extruder KCC2 is expressed late in development, whereas NKCC1, which accumulates chloride in the cell, is more expressed in immature neurons. Reprinted from "GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations", by Y. Ben-Ari, 2007, *Physiological Reviews*, 87(4): 1214-1284.

4.3.2.2.2. Early generated patterns of neuronal activity

Early generated activities are physiological patterns that are conserved across species. These synchronous activities, generated during critical periods for synaptogenesis and maturation, are associated to an increase in $[Ca^{2+}]_i$. In immature neurons, the activation of $GABA_A$Rs induces a transient increase in membrane conductance, leading to an increase in $[Ca^{2+}]_i$ via activation of voltage-gated Ca^{2+} channels[434]. This increase enables signal transduction and modulation of synaptic activity. Altogether, these early generated patterns contribute to the structural refinement of neural circuits. These patterns have been described in various structures such as the cortex (early network oscillations)[435], and the retina (waves)[436]. In the hippocampus, two types of early patterns have been identified: synchronous plateau assemblies (SPAs) and giant depolarizing potentials (GDPs).

SPAs are non-synaptic long-lasting spontaneous activities generated by small cell assemblies coupled by gap junctions. They appear during embryonic life with a peak at birth, and prior to the occurrence of GDPs, even though GPDs and SPAs shortly coexist within the same neuronal network during the first postnatal week (Figure 14A). During this stage, SPAs expression is progressively inhibited by synaptic-driven activities that are GDPs. SPAs generation requires the activation of the hyperpolarization-activated cationic current Ih, as well as sodium voltage-gated and Ca^{2+} L-type voltage-gated channels[437]. In addition, SPAs generation is modulated by oxytocin (OT) and $GABA_A$R actions. Indeed, OT treatment increases the number of cells with SPAs in embryonic life while OTR antagonist treatment at birth decreases this number (Figure 14B). Moreover, GABA depolarizing activity during fetal and postnatal life inhibits SPAs emergence, since blockade of $GABA_A$Rs increases the number of cells with SPAs (Figure 14C). Hence, the oxytocin-mediated transient inhibitory action of GABA at birth favor the emergence of SPAs[437].

In the hippocampus, GDPs represent the first synchronous spontaneous synaptic activity and consists in slow polysynaptic events separated by quiescent intervals[438]. They enable the translation of specific patterns of pre- and postsynaptic activity into long-lasting changes in synaptic strength, and the stabilization of synaptic connections[439]. GDPs appear during early postnatal life and disappear after P12[438], and their genesis is dependent on the depolarizing and excitatory actions of both glutamate and GABA[439]. Yet, the mechanisms involved are not clear and three mechanisms have been proposed to participate in GDPs generation. (1) CA3 pyramidal cells play a “pacemaker” role associated to persistent sodium currents that are

activated by $GABA_AR$ tonic current-mediated membrane depolarization[440]. (2) Summation of postsynaptic potentials generated from different initiation sites, beyond threshold, generate GDPs[441]. (3) Among interneurons, a subpopulation of GABA-releasing interneurons are superconnected nodes called hub neurons that, by orchestrating network synchronization, influence the entire network dynamics[442].

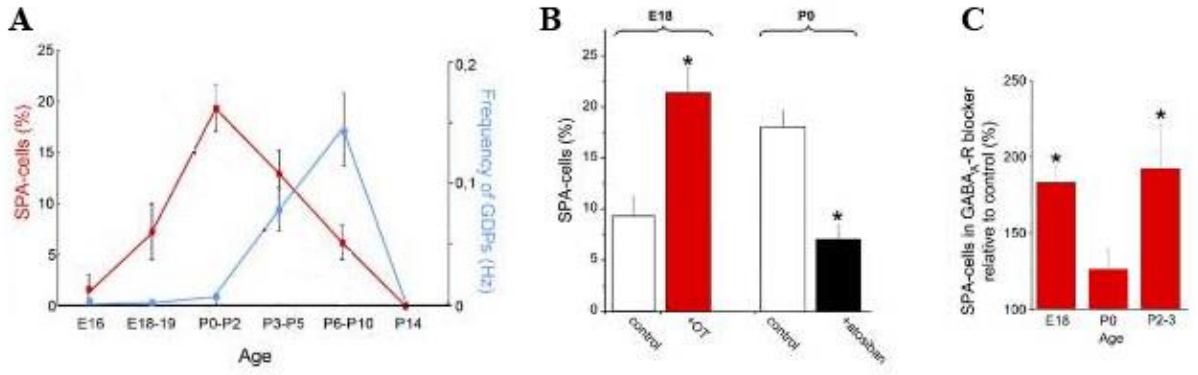


Figure 14. Developmental features of SPAs. (A) Fraction of SPA-cells (red) relative to the number of active cells, and frequency of GDPs (blue) for six successive age groups. (B) Averaged fraction of active cells producing a calcium plateau (fraction of SPA-cells) at E18 and P0 in control conditions, in the presence of OT (1µM), and in the presence of atosiban (OTR antagonist, 1-5µM). (C) Fraction of SPA cells in the presence of the $GABA_AR$ blocker (bicuculline 10µM) relative to control conditions. Adapted from "A parturition-associated nonsynaptic coherent activity pattern in the developing hippocampus", by V. Crépel et al., 2007, *Neuron*, 54(1): 105-120.

4.3.2.2.3. Neuronal maturation

During development, GABA actions also include regulation of proliferation, migration, growth, and synapse formation[429,443]. These trophic effects are mediated by $GABA_AR$ activation and GABA depolarizing effect during early development that result in increased intracellular Ca^{2+} concentrations[444]. Ca^{2+} is a conventional second messenger that activates numerous pathways including neuronal maturation pathways. Indeed, in hippocampal cultures, activation of $GABA_AR$ by its agonist muscimol increases the soma area and number of NPY-positive interneurons. In addition, these effects might be mediated through BDNF, as $GABA_AR$ activation increases BDNF mRNA levels, and BDNF induces similar results as those seen with muscimol[445,446] (Figure 15). Hence, $GABA_AR$ stimulation, by inducing BDNF release, regulates the morphology of hippocampal interneurons.

In addition to its action via $GABA_ARs$, GABA also binds to metabotropic $GABA_BRs$ to promote GABAergic synapses maturation. In early development, $GABA_BRs$ are activated by the simultaneous release of GABA from several interneurons[447], hence requiring the presence of spontaneous GDPs[432]. Similarly to $GABA_AR$, $GABA_BR$ triggers BDNF release in hippocampal neurons cultures, encouraging functional maturation of GABAergic synapses[432].

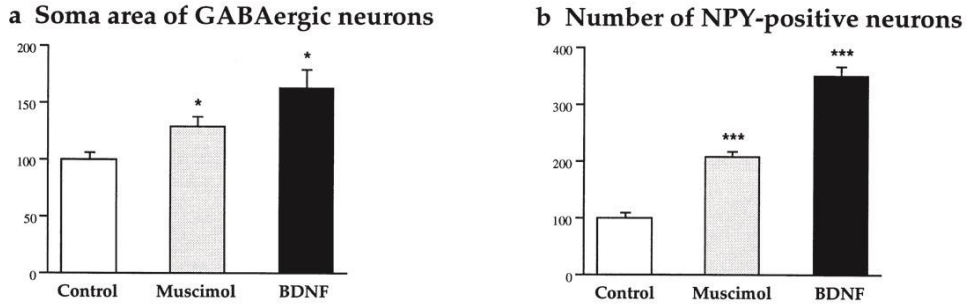


Figure 15. Muscimol and BDNF promote the differentiation of GABAergic neurons after one week in vitro. Effects of 100µM muscimol and 20ng/ml BDNF on (a) the area the soma of GABAergic neurons, (b) the total number of cells. Reprinted from "GABAergic stimulation regulates the phenotype of hippocampal interneurons through the regulation of brain-derived neurotrophic factor", by S. Marty et al. 1996, *Neuron*, 16(3): 565-570.

Therefore, in early development, GABA promotes BDNF release via activation of ionotropic $GABA_A$Rs and metabotropic $GABA_B$Rs, hence promoting GABAergic interneurons maturation.

RESULTS

My thesis work aimed at further understanding if C-section delivery had an influence on the risk of developing ASD, if this risk was different according to the gestational age when the C-section was performed, and the biological characteristics that might be associated to C-section delivery and ASD.

I focused on the GABAergic system and its relationship with ASD by relying on previous studies done in our lab to test if C-section delivery might induce ASD. The studies of our lab showed that the oxytocin-mediated GABA hyperpolarizing shift at birth is altered in the hippocampus (CA3) of two rodent models of autism[127]. Also, the GABA developmental shift from excitation to inhibition (E to I) during the second postnatal week is abolished, hence a persistent depolarizing GABA effect in hippocampal CA3 pyramidal cells is present in ASD rodent offspring. The restoration of the transient hyperpolarizing GABA shift at birth by the administration of bumetanide (NKCC1 antagonist) leads to the restoration of the postnatal GABA switch from E to I in CA3 pyramidal cells[127].

Based on these observations, we hypothesized that a disruption of the mode and time of delivery might lead to an alteration of the GABA developmental sequence in CA3 pyramidal cells and induce a persistent depolarizing effect in those cells such as the one observed in two models of autism. To test this hypothesis, we implemented a mouse model of C-section delivery at term and preterm, and assessed the physiological and behavioral features in the offspring.

Term or Preterm Cesarean Section Delivery Does Not Lead to Long-term Detrimental Consequences in Mice

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ORIGINAL ARTICLE

Term or Preterm Cesarean Section Delivery Does Not Lead to Long-term Detrimental Consequences in Mice

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Abstract

Epidemiological studies have provided contradictory data on the deleterious sequels of cesarean section (C-section) delivery and their links with developmental brain disorders such as Autism Spectrum Disorders. To gain better insight on these issues, we have now compared physiological, morphological, and behavioral parameters in vaginal, term, and preterm C-section delivered mice. We report that C-section delivery does not lead to long-term behavioral alterations though preterm C-section delivery modifies communicative behaviors in pups. Moreover, C-section delivery neither alters the gamma-aminobutyric acid (GABA) developmental excitatory to inhibitory shift nor the frequency or amplitude of glutamatergic and GABAergic postsynaptic currents in hippocampal pyramidal neurons. However, these neurons present an underdeveloped dendritic arbor at birth in pups born by C-section delivery, but this difference disappears one day later suggesting an accelerated growth after birth. Therefore, C-section delivery, with prematurity as an aggravating factor, induces transient developmental delays but neither impacts the GABA developmental sequence nor leads to long-term consequences in mice. The deleterious sequels of C-section delivery described in epidemiological studies might be due to a perinatal insult that could be aggravated by C-section delivery.

Key words: autism spectrum disorders, birth, GABA, prematurity

Introduction

Delivery is amongst the most complex biological processes in mammals. Numerous vital changes must take place in a relative short period of time, including the transition from a liquid medium to an aerial one (Bland et al. 1982, Hooper, Te Pas, and Kitchen 2016), a shift from continuous to intermittent nutrient supply (Ward Platt and Deshpande 2005), a drop in body temperature (Jaykka and Laakso 1967), and the release of hormones such as catecholamines, glucocorticoids, and oxytocin which are required to facilitate lungs maturation and prevent neuronal hyperactivity in the newborn (Lagercrantz and Slotkin 1986, Mazzuca et al. 2011, Hillman, Kallapur, and Jobe 2012). Still, our understanding of neuronal activity before, during, and shortly after delivery is very limited. In addition, perinatal alterations have been associated with an increased incidence of neurodevelopmental disorders (Chudal et al. 2014, Brander et al. 2016, 2017), making birth a highly vulnerable period. This stresses the clinical relevance of determining the sequence of events occurring around birth, and how deviations to this sequence could lead to the development of disorders.

An important birth modification is cesarean section (C-section) delivery, whose incidence has continuously increased for the last 20 years and is now estimated to be 18.6% worldwide, with peaks above 50% in countries such as Brazil, Dominican

Republic, and Egypt (Betran et al. 2016). Furthermore, the incidence of preterm birth has also increased over the past 20 years with 12% of babies born prematurely in average in low income countries (compared with 9% in high-income countries) (WHO 2017b). Several factors have been suggested to contribute to this rise in preterm birth, including changes in obstetric practices associated with a higher number of preterm C-section deliveries (WHO 2017b).

Epidemiological studies suggest that C-section delivery might lead to a higher incidence of neurodevelopmental brain disorders, notably Autism Spectrum Disorders (ASD; Glasson et al. 2004, Al-Ansari and Ahmed 2013, Curran et al. 2015, Yip et al. 2017). These assumptions are however disputed by other studies (Bilder et al. 2009, Zhang et al. 2010). Such contradictions might be due to the complexity of parameters associated with C-section delivery including emergency versus elective C-section, maternal and fetal indications (placenta previa, obstructed labor, severe preeclampsia, …), use of anesthetics, gestational age, etc.

Despite their limitations when compared with humans, rodent studies offer the possibility to test in well-controlled conditions the relationship between term or preterm C-section delivery and brain disorders. Experimental studies suggest that pups born by C-section delivery present, as in humans, mild respiratory distress, and reduced plasma catecholamine and glucocorticoids levels in comparison with vaginally born pups (Usher, Allen, and McLean 1971, Boksa and Zhang 2008). In addition, an alteration in dopamine (DA) levels under stress conditions and long-term changes in DA-modulated behaviors have also been reported (Vaillancourt and Boksa 2000, Boksa and El-Khodor 2003). Nonetheless, C-section delivery at term has been shown not to alter the initiation of the barrel cortex formation which is triggered at birth (vaginally either at term or preterm) (Toda et al. 2013).

Here, we tested the hypothesis that C-section delivery alters developmental sequences leading to neurodevelopmental disorders, notably ASD. Because of the paucity of experimental information available, we screened a variety of behavioral features in mice delivered at term (0-6h before expected birth) or preterm (6-24h before expected birth). In addition, we determined physiological features including alterations of GABAergic signaling since an imbalance between excitation and inhibition has been related to autism (Rubenstein and Merzenich 2003, Ben-Ari 2014, Tyzio et al. 2014). Moreover, gamma-aminobutyric acid (GABA) follows a developmental sequence with a transient oxytocin-mediated hyperpolarizing shift during delivery (Tyzio et al. 2006), and a permanent excitatory to inhibitory (E-I) shift during the second postnatal week in rodents (Ben-Ari et al. 2007). These shifts have been shown to be abolished in CA3 hippocampal neurons in two rodent models of autism (Tyzio et al. 2014). The restoration of the oxytocin-mediated GABA inhibition at birth by the NKCC1 antagonist bumetanide led to the recovery of the E-I GABA shift 2 weeks later and the attenuation of their autism pathogenesis (Eftekhari et al. 2014, Tyzio et al. 2014). These results highlight the importance of GABA signaling as a major factor in the development of autistic-like disorders.

We report that C-section delivery at term or preterm in mice neither leads to major long-term autistic-like behaviors in adulthood nor to altered GABA and glutamate signaling during the second postnatal week. The deleterious effects are restricted to the early neonatal period with communicative impairments in preterm C-section pups, and a transient underdevelopment of the dendritic arborization of CA3 pyramidal neurons at birth after C-section delivery. Therefore, our results suggest that C-section delivery induces transient developmental delays that do not lead to long-term consequences.

Material and Methods

Animals

Two strains of mice were used: NIHS mice (Envigo, UK) and RjOrl: Swiss mice (Janvier, France). All experiments were performed in accordance with the European Communities Council Directive (2010/63/EU). Mice were maintained on a 12-h light cycle (7AM-7PM) with *ad libitum* access to food and water. Matings were done overnight by placing one male with two females. Vaginal plugs were checked early the following morning at 7AM and noted as E0.5 day of gestation.

Cesarean Section Procedure

C-sections were performed on timed-pregnant mice after 6PM for term C-sections, in a time window of maximum 6 h before estimated birth; and after 7PM for preterm C-sections, in a time window of 6-24 h before estimated birth. C-sections were performed under aseptic conditions using a modification of the procedure described by El-Khodor and Boksa (1997). In order to avoid the use of anesthetics, pregnant dams were euthanized by cervical dislocation and the uterine horns were rapidly isolated from their blood supply (in 20-30s). The pups were delivered and placed on a heating pad (34-35°C) for 15 min where they were softly massaged by using q-tips to remove the liquid from their lungs and stimulate breathing. Immediately after, they were given to a surrogate mother.

Slice Preparation

Electrophysiological experiments were performed on mice of both sexes from P14 to P16. Animals were euthanized by decapitation and their brain rapidly removed and placed in ice-cooled choline with the following composition (in mM): 132.5 choline chloride, 2.5 KCl, 1.23 $NaH_2PO_4.H_2O$, 25 $NaHCO_3$, 8 glucose, 0.7 $CaCl_2$, 3 $MgCl_2$, pH 7.4 equilibrated with 95% O_2

and 5% CO_2. Horizontal hippocampal slices (400 µm thick) were cut using a vibratome (Leica-VT1200S, Leica Biosystems, Germany) and kept in oxygenated artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 126 NaCl, 3.5 KCl, 1.2 $NaH_2PO_4.H_2O$, 11 glucose, 25 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgCl_2$, pH 7.4 equilibrated with 95% O_2 and 5% CO_2 for at least 1h before use. Slices were placed into the recording chamber where they were fully submerged and superfused with oxygenated ACSF at a rate of 2-3 mL/min at room temperature (22-25°C).

Electrophysiological Recordings

Cell-Attached Recordings

Recordings of single $GABA_A$ receptor channels in cell-attached configuration were performed in CA3 pyramidal cells using an EPC-10 amplifier (HEKA Elektronik Dr Schulze GmbH, Germany). Patch pipette solution contained (in mM): 120 NaCl, 5 KCl, 20 TEA-Cl, 5 4-aminopyridine, 0.1 $CaCl_2$, 10 $MgCl_2$, 10 glucose, 10 HEPES-NaOH, pH 7.2-7.3 (with GABA at 5µM). Recordings were digitized and analyzed as described previously (Khazipov, Ragozzino, and Bregestovski 1995, Tyzio et al. 2003).

Extracellular Field Potentials and Multi-Unit Activity Recordings

Extracellular field recordings were performed in the CA3 pyramidal layer of acute hippocampal slices by using glass pipettes (Harvard Apparatus, MA, USA) containing ACSF. Isoguvacine (10 µM; Sigma-Aldrich, MO, USA), a $GABA_A$ receptor agonist, was applied for 90s. Signals were recorded with a low-noise multichannel DAM-80A amplifier (WPI, UK; low-pass filter 1Hz; high-pass filter 3 kHz; gain x1000) and digitized online with a Digidata 1400A digitizer (Molecular Devices, CA, USA). Analysis of synaptic activities was done using Clampfit 10.4 software (Molecular Devices, CA, USA). The spike detection threshold was defined as three times the standard deviation of the noise recorded in the bath solution. Spike frequency was calculated for control, isoguvacine and wash-out periods. Wash-out spike frequency that did not come back to control levels (±10% of control) was a criterion of exclusion of slices.

Whole-Cell Recordings

Whole-cell recordings of CA3 pyramidal neurons were performed using a MultiClamp 700B amplifier (Molecular Devices, CA, USA). Data were low-pass filtered at 2.4 kHz and acquired using a Digidata 1550A (Molecular Devices, CA, USA). Patch pipette solution contained (in mM): 130 K-gluconate, 10 Na-gluconate, 7 NaCl, 4 Mg-ATP, 10 HEPES, 4 phosphocreatine, 0.3 Na-GTP, pH 7.3 with KOH. Spontaneous GABAergic postsynaptic currents (sIPSCs) were recorded for 15 min at the reversal potential for glutamatergic currents (+5mV), and spontaneous glutamatergic postsynaptic currents (sEPSCs) were recorded for 10 min at the reversal potential for GABAergic currents (-70mV). sIPSCs and sEPSCs were analyzed using Clampfit 10.4 (Molecular Devices, CA, USA) and Mini Analysis 6.0.7 (Synaptosoft Inc, NJ, USA) softwares.

Biocytin-Filled Hippocampal Pyramidal Neuron Morphology

CA3 pyramidal cells were filled with biocytin (Sigma-Aldrich, MO, USA) for 10 min from P0.5 to P2.5 in pups of both sexes. Slices were fixed overnight at 4°C with Antigenfix (Microm Microtech, France), rinsed with phosphate buffered saline (PBS) and saturated in PBS containing 0.3% Triton X-100 (PBST; Sigma-Aldrich, MO, USA) and 5% normal goat serum (PBST-NGS; Thermo Fisher Scientific, MA, USA). Slices were incubated overnight at 4°C in Alexa Fluor™ 555-conjugate streptavidin (1/1000; Thermo Fisher Scientific, MA, USA) in PBST-NGS. Then, they were rinsed with PBS, incubated with Hoechst (Sigma Aldrich, MO, USA) for 10 min and rinsed with PBS. Slices were mounted with Fluoromount-G (Electron Microscopy Sciences, PA, USA). The acquisition of the stained neurons was performed with a confocal microscope (Leica SP5X, Wetzlar, Germany) and the analysis was done with Fiji (open-source platform)(Schindelin et al. 2012). Criteria used to include pyramidal neurons in the morphometric analysis were the good quality of intracellular loading and the presence of only one apical primary dendrite.

Behavior

Ultrasonic Vocalizations

Isolation-induced ultrasonic vocalizations (USVs) were recorded in pups of both sexes at P9 to test early communication behaviors as described previously (Tyzio et al. 2014). In brief, after 30 min of habituation to the testing room, neonatal mice were individually isolated from their mother and placed in an isolation box (23 x 28 x 18 cm) located inside an anechoic box (54 x 57 x 41 cm; Couldbourn instruments, PA, USA) for a 3 min test. An ultrasound microphone (Avisoft UltraSoundGate condenser microphone capsule CM16/CMPA, Avisoft bioacoustics, Germany) sensitive to frequencies of 10-250 kHz was located in the roof of the isolation box. Recordings were done using Avisoft recorder software (version 4.2) with a sampling rate of 250 kHz in 16 bit format. Data were transferred to SASLab Pro software (version 5.2; Avisoft bioacoustics) and a fast Fourier transformation was conducted (512 FFT-length, 100% frame, Hamming window, and 75% time window overlap) before the analysis. Recordings were analyzed for the number of calls, total calling time, and call duration.

Three-Chamber Social Test

Young-adult male mice from P55 to P60 were tested for sociability and social novelty preference as previously described by Desbonnet et al. (2014). Briefly, after 30 minutes of habituation to the testing room, mice were placed in a rectangular apparatus (59 x 39.5 cm; Noldus,

Netherlands) divided in three chambers (19.5 x 39.5 cm) by transparent partitions with small openings allowing easy access to all compartments. The test was composed of three 10 min trials where the tested mouse could explore freely all three chambers. The first trial consisted of the habituation of the tested mouse to the apparatus. For the second session (sociability test), an unfamiliar mouse of the same sex and age was placed into a wire cage either in the left or right chambers. For the final session (social novelty preference), a second unfamiliar mouse was placed in the chamber opposite to the now-familiar mouse. Behaviors were recorded by a video camera placed on a bar above the apparatus by using the Ethovision XT software (Noldus, the Netherlands) and analyzed manually for the time spent in each of the chambers.

Grooming

Young-adult male mice 8-9 weeks old were used for the evaluation of grooming behavior. After 30 min of habituation to the testing room, mice were individually placed in a plexiglass cylinder (15 x 45 cm; Form X.L., France) and video-taped by a camera placed on a bar above the apparatus for 20 min. The first 10 min of the test were used to habituate the mouse to the cylinder and the last 10 min for the actual test. Recordings were done with the Ethovison software (Noldus, the Netherlands) and analyzed manually for the time spent grooming, the number of grooming events and the latency to start grooming. Grooming behaviors were defined as described previously by Pobbe et al. (2012).

Open Field

Young-adult male mice 8-9 weeks old were tested for locomotor and anxiety-like behaviors in an open field arena. After 30 min of room habituation, mice were individually placed in a square apparatus (40 x 40 cm; Noldus, the Netherlands) for 10 min of testing. Behaviors were recorded by a video camera above the apparatus and the automated tracking system Ethovison XT (Noldus, the Netherlands) was used to analyze the data. Anxiety-like behavior was defined by the time spent in the center compared with the time spent in the periphery (thigmotaxis), by the number of entries in the center, and the latency to enter the center of the open field arena. Locomotor activity was assessed by the velocity, the total distance traveled, and the moving time of mice during the test.

Statistics

Data for cell-attached recordings were analyzed with two-tailed *t*-test. Extracellular field potential data were analyzed by the repeated measures one-way ANOVA with Dunnett's multiple comparisons post hoc test. Spontaneous glutamatergic and GABAergic activities were analyzed for the frequency by one-way ANOVA with Tukey's post hoc test and for the amplitude by Kruskal-Wallis with Dunn's post hoc test. For social behavior, the Mann-Whitney test was used to compare empty versus unfamiliar and now-familiar versus novel conditions within the same group. For grooming, open field, morphological metrics and USVs, Kruskal-Wallis with Dunn's post hoc test was used to compare data between each groups. All data are presented as mean ± standard error of the mean (SEM).

Results

Cesarean Section Delivery Does Not Alter Social Behavior in Adulthood

The three-chamber test was used to assess sociability and social novelty in young-adult male mice born vaginally or by C-section delivery. Social behavior, assessed by a higher time spent in the unfamiliar mouse chamber compared to the time spent in the empty chamber, was seen for vaginal (314 ± 12.25 s vs 181 ± 9.91 s; $p<0.0001$), term C-section (299.9 ± 15.47 s vs 189 ± 12.93 s; $p<0.0001$) and preterm C-section mice (271.2 ± 14.88 s vs 208 ± 10.66 s; $p=0.0027$; Figure 1A-B, Supplementary Table 1). In the second part of the test, mice exhibited no social novelty as the time spent in the now-familiar mouse chamber vs the time spent in the novel mouse chamber was similar for vaginal (225.6 ± 9.87 s vs 245.2 ± 9.39 s; $p=0.1782$), term C-section (219 ± 13.15 s vs 247.1 ± 13.63 s; $p=0.0687$) and preterm C-section mice (228.3 ± 10.09 s vs 233.9 ± 10.79 s; $p=0.3211$; Figure 1C-D, Supplementary Table 1). Therefore, mice born by C-section delivery are not socially different from vaginal-born mice.

Term Cesarean Section Delivery Produces Minor Alterations of Stereotypic Behaviors in Adulthood

Self-grooming was used to assess stereotypic behaviors by measuring the time spent grooming, the number of grooming events and the latency to start grooming in young-adult male mice. Mice born at term by C-section spent more time grooming (163.7 ± 13.39 s) than vaginal mice (114.9 ± 10.95 s; $p=0.0219$), whereas preterm C-section mice spent as much time grooming (121.8 ± 15.99 s) than vaginal and term C-section mice (Figure 1E, Supplementary Table 2). However, the number of grooming events was similar for vaginal (8.52 ± 0.88), term C-section (7.82 ± 0.72) and preterm C-section mice (9.47 ± 1.42; Figure 1F, Table S2), and the latency to start grooming did not differ between vaginal (72.55 ± 12.1 s), term C-section (75.77 ± 16.69 s) and preterm C-section mice (53.12 ± 17.22 s; Figure 1G, Supplementary Table 2). Hence, term C-section mice show a mild increase in stereotypic behavior whereas preterm C-section mice show no alteration of this behavior.

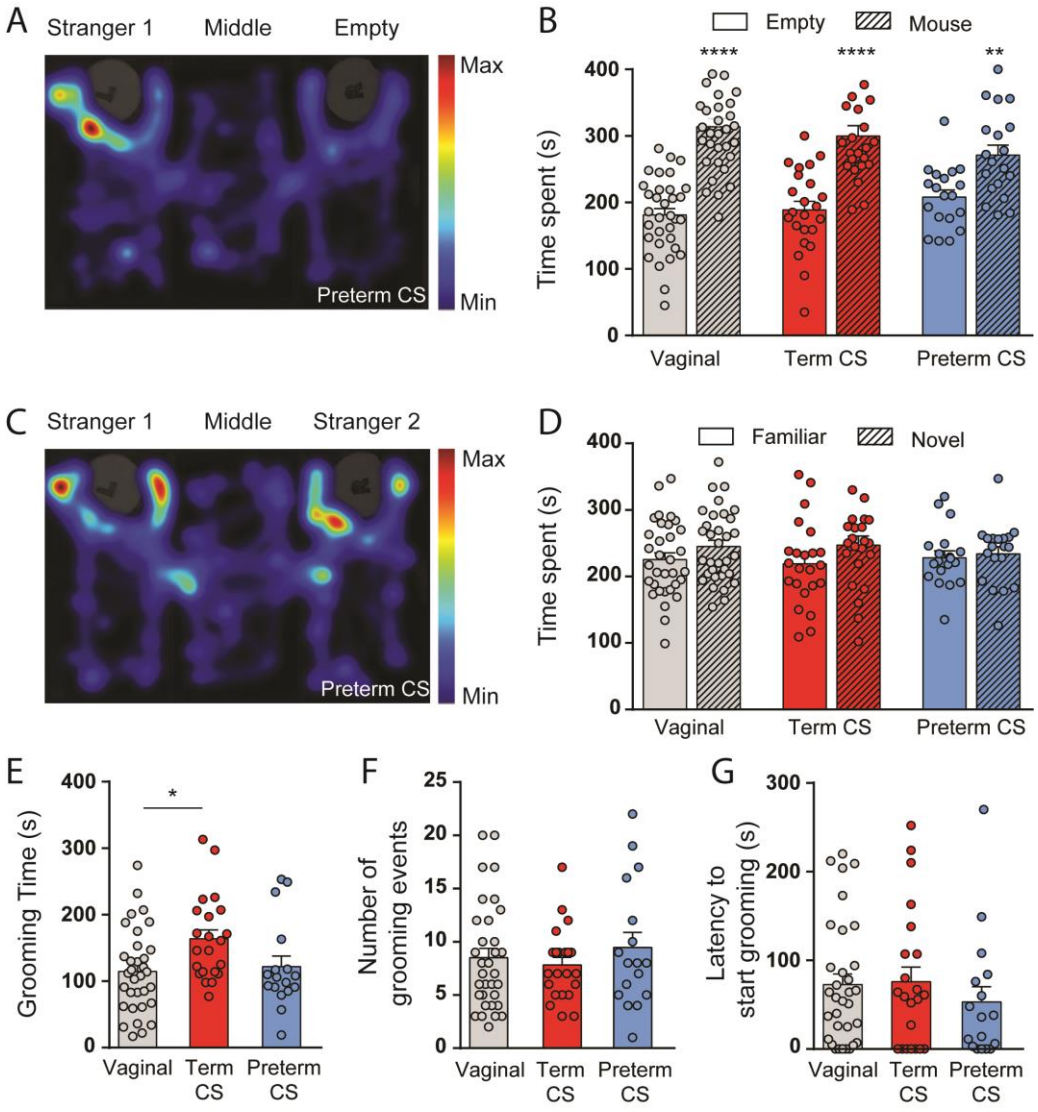

Figure 1. Effects of term or preterm cesarean section (CS) delivery on autistic-like behaviors in young-adult male mice. (A) Representative heatmap of a preterm CS mouse movements in the sociability test. (B) Sociability is represented by the time the mouse spent in the chamber with the unfamiliar mouse vs the empty chamber. (C) Representative heatmap of a preterm CS mouse movements in the social novelty test. (D) Social novelty test is represented by the time the mouse spent in the chamber with the now-familiar mouse vs with a novel mouse. Grooming behavior was assessed for vaginal, term CS and preterm CS mice by (E) the time spent grooming, (F) the grooming frequency, and (G) the latency to start grooming. Data are presented as mean ± SEM, *p<0.05, **p<0.01, ****p<0.0001. (B and D) n=33 for vaginal, n=23 for term CS and n=19 for preterm CS. (E, F and G) n=33 for vaginal, n=22 for term CS and n=17 for preterm CS.

Term and Preterm Cesarean Section Delivery Do Not Alter Locomotor Nor Anxiety-Like Behavior in Adulthood

Open field was used to assess both anxiety-like and locomotor behaviors in young-adult male mice. To assess anxiety, we measured the time mice spent in the center of an open field arena. Term and preterm C-section mice spent a similar amount of time in the center (49.29 ± 3.77 s and 39.04 ± 3.28 s respectively) as vaginal mice (45.71 ± 3.6 s; Figure 2A-B, Supplementary Table 3). The number of entries in the center (Figure 2C, Supplementary Table 3) was also similar between vaginal (47.64 ± 2.5), term C-section (46.26 ± 2.88) and preterm C-section mice (42.84 ± 3.34). Finally, the latency to first enter the center of the open field (Figure 2D, Supplementary Table 3) was not statistically different for vaginal (12.76 ± 2.61 s), term C-section (16.92 ± 4.29 s) and preterm C-section mice (13.64 ± 3.42 s). Therefore, mice born by C-section do not show anxiety-like behavior.

To characterize locomotor activity, we assessed three parameters: the velocity, the distance traveled, and the time mice spent moving. The velocity (Figure 2E, Supplementary Table 4) was similar for vaginal (7.18 ± 0.3 cm/s), term C-section (6.55 ± 0.2 cm/s) and preterm C-section mice (6.49 ± 0.37 cm/s). The total distance traveled (Figure 2F, Supplementary Table 4) was not

statistically different for vaginal (4301 ± 177.5 cm), term C-section (3919 ± 120.5 cm) and preterm C-section mice (3893 ± 220.5 cm). The same observation was made for the time spent moving (Figure 2G, Supplementary Table 4) as this parameter was not different between vaginal (440.7 ± 8.5 s), term C-section (419.2 ± 9.02 s) and preterm C-section mice (408.6 ± 10.44 s). These results show that C-section delivery does not induce an alteration in the locomotor behavior of mice.

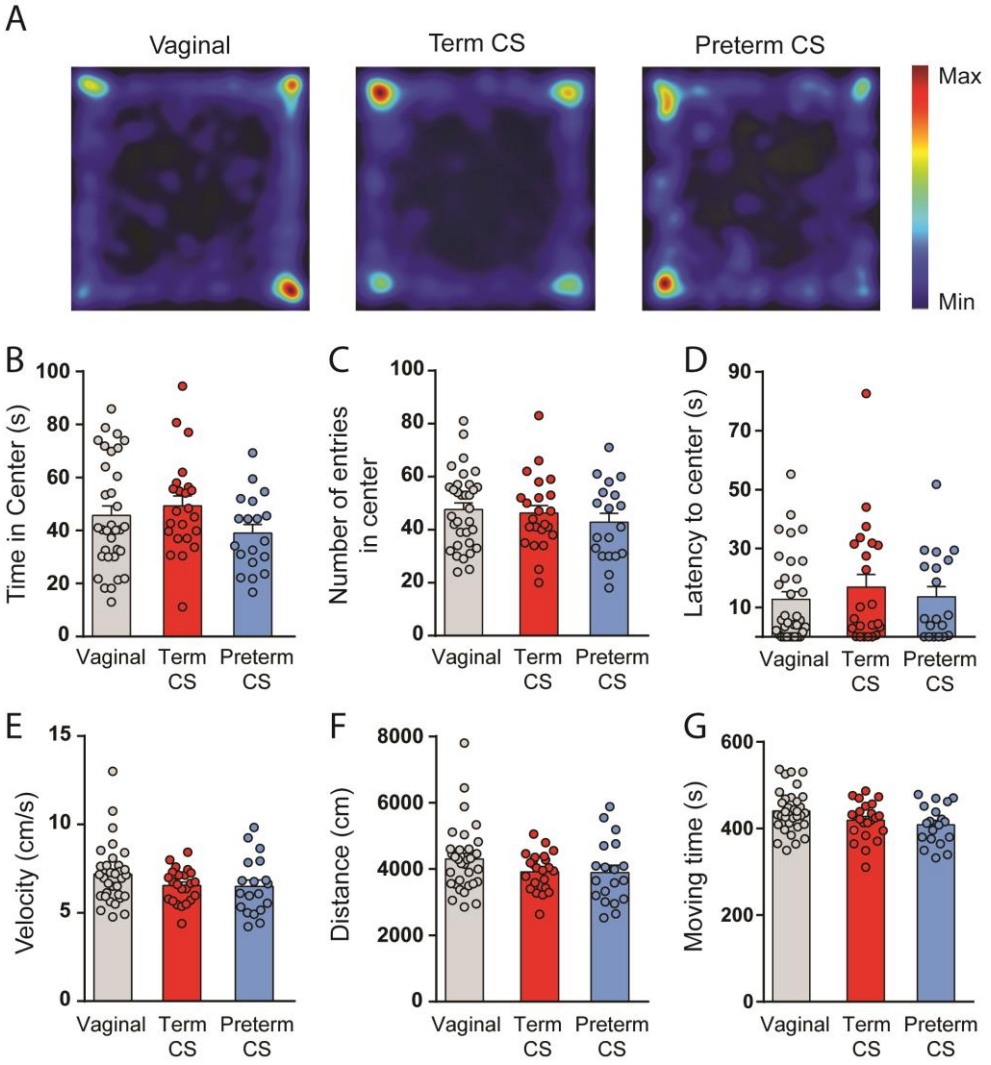

Figure 2. Locomotor and anxiety-like behaviors in young-adult male mice are not affected by term or preterm CS delivery. (A) Representative heatmap of the movement of mice during the open field test. Anxiety-like behaviors were compared between vaginal, term CS and preterm CS mice with (B) the time spent in the center of the arena, (C) the number of entries in the center, and (D) the latency to enter the center for the first time. Locomotor activities were determined in mice by (E) the velocity, (F) the total distance traveled, and (G) the time spent moving. Data are presented as mean ± SEM, (B-G) n=33 for vaginal, n=23 for term CS and n=19 for preterm CS.

Cesarean Section Delivery Alters Communicative Behaviors of Pups

Isolation-induced USVs were evaluated at P9 to assess early communicative alterations. Preterm C-section pups presented a higher number of calls than vaginal pups (206.7 ± 29.25 s vs 89.05 ± 14.14 s; p=0.0013; Figure 3A) as well as a higher total calling time (8.17 ± 1.27 s vs 3.24 ± 0.55 s; p=0.0011; Figure 3B). No differences were seen between vaginal and term C-section pups for these two parameters (Figure 3A-B and Supplementary Table 5). The call duration was similar for term C-section (34.48 ± 1.24 ms), preterm C-section (36.15 ± 1.23 ms) and vaginal pups (32.44 ± 1.16 ms; Figure 3C and Supplementary Table 5). Therefore, preterm C-section delivery is associated with USVs modifications in pre-wean mice.

Cesarean Section Delivery Does Not Affect the GABA Excitatory to Inhibitory Shift

To examine whether the action of GABA was altered after C-section delivery, cell-attached recordings were performed and the driving force of $GABA_A$ receptors

(DF_{GABA}) recorded. At P14-P15, neurons from term and preterm C-section born pups presented a similar DF_{GABA} (4.7 ± 5.8 mV and 2.6 ± 2.9 mV respectively), with no difference compared to vaginal CA3 pyramidal neurons (4.1 ± 3.6 mV; p=0.226 and p=0.357 respectively; Figure 4A-B, Supplementary Table 6). To confirm the physiological effect of GABA polarity, extracellular field potential recordings were performed at P15-P16. Isoguvacine was applied on hippocampal slices to determine whether GABA had an excitatory or an inhibitory effect. Isoguvacine significantly decreased the spike frequency in hippocampal slices of vaginal (78.89 ± 2.71% of control; p=0.0001), term C-section (73.92 ± 5.47%; p=0.0009) and preterm C-section mice (79.55 ± 3.23%; p=0.0001; Figure 4C-F; Supplementary Table 7). Therefore, CA3 pyramidal neurons of pups born vaginally or after C-section exhibit an inhibitory GABA with a similar polarity at P14-P16.

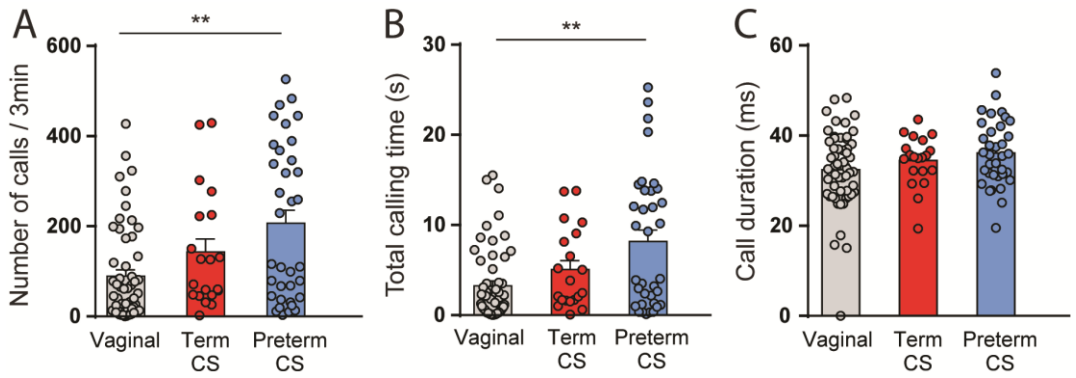

Figure 3. Preterm CS delivery alters early communicative behaviors in mice. Effect of CS delivery on isolation-induced ultrasonic vocalizations at P9 for (A) number of calls, (B) total calling time, and (C) call duration. Data are presented as mean ± SEM, **p<0.01. (A-C) n=55 for vaginal, n=20 for term CS and n=35 for preterm CS.

Cesarean Section Delivery Does Not Alter Spontaneous Glutamatergic and GABAergic Activities

Network activities were assessed in CA3 pyramidal neurons with whole-cell patch clamp recordings. At P14-P15, the amplitude and frequency of sEPSCs (Figure 5A-C, Supplementary Table 8) were similar for neurons from vaginal (15.76 ± 0.94 pA; 24.93 ± 1.18 Hz), term C-section (17.01 ± 1.37 pA; 22.88 ± 1.45 Hz) and preterm C-section mice (13.85 ± 1.06 pA; 21.87 ± 1.68 Hz). Furthermore, no difference was seen in the amplitude and frequency of sIPSCs (Figure 5D-F, Supplementary Table 8) in neurons from vaginal (72.02 ± 2.81 pA ; 9.68 ± 0.63 Hz), term C-section (66.57 ± 1.69 pA; 9.84 ± 0.72 Hz) and preterm C-section mice (68.82 ± 3.26 pA; 8.39 ± 0.97 Hz). Thus, C-section delivery does not affect the glutamatergic and GABAergic network activities in CA3 pyramidal neurons at P14-P15.

Cesarean Section Delivery Transiently Delays CA3 Pyramidal Neurons Apical Dendrite Arborization

To evaluate if the morphological properties of CA3 pyramidal neurons were altered after birth by C-section delivery, neurons were filled with biocytin (Figure 6A) and reconstructed for morphological analysis (Figure 6B,C). We observed that at P0.5, the total dendritic length is shorter for pups born by C-section delivery at term (573 ± 42.04 µm; p=0.0218) or preterm (537.2 ± 49.43 µm; p=0.004) compared to vaginally born pups (784.6 ± 58.83 µm; Figure 6E). The total number of intersections is also lower in neurons of pups born by C-section at term or preterm (419.9 ± 30.52 µm; p=0.0265; and 370.8 ± 31.21 µm; p=0.0017 respectively) compared to vaginal ones (563.1 ± 43.05 µm; Figure 6H,I). In addition, the primary dendritic length was shorter for preterm neurons (10.39 ± 2.09 µm) compared to term C-section (19.04 ± 1.92 µm; p=0.0003) and vaginal neurons (19.72 ± 2.88 µm; p=0.0054; Figure 6D). Finally, the ending radius and critical radius were shorter for preterm C-section delivery compared to vaginal neurons (99.9 ± 4.73 µm vs 130.4 ± 5.11 µm; p=0.0004 and 59.51 ± 4.90 µm vs 86.61 ± 5.63 µm; p=0.0022 respectively; Figure 6F,G, Supplementary Table 9). However, no difference could be seen between the three groups for all these parameters at P1.5 and P2.5 (Figure 6D-I, Supplementary Table 10 and 11). Thus, C-section delivery transiently alters the neuronal growth of CA3 pyramidal neurons at P0.5, a phenotype which is aggravated by the gestational age at the time of birth.

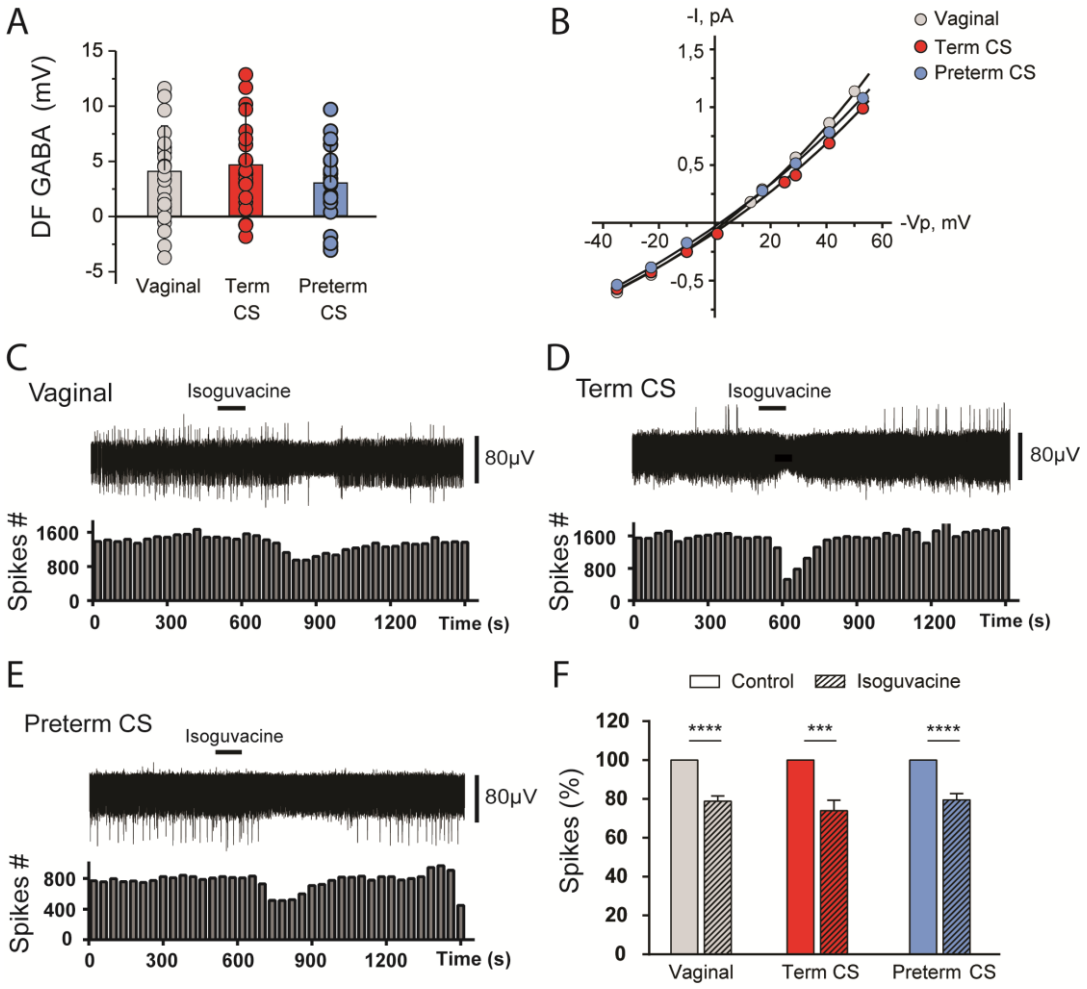

Figure 4. Inhibitory action of GABA in CA3 pyramidal neurons of pups born by term or preterm CS around postnatal age P14. (A) Average values of DF_{GABA} measured in hippocampal CA3 pyramidal neurons from P14 to P15. (B) Representative I/V curves from cell-attached recordings for vaginal, term CS and preterm CS groups. (C-E) Effect of isoguvacine (10µM; black bar) on representative traces of spontaneous extracellular field potentials from P15 to P16 with corresponding time courses of spike frequency changes for vaginal (C), term CS (D) and preterm CS (E). (F) Histogram of the averaged normalized spike frequency in control and isoguvacine (hatched bars) periods. for vaginal, term CS and preterm CS mice. Data are presented as mean ± SEM, ***p<0.001, ****p<0.0001. (A) n=31 for vaginal, n=19 for term CS and n=15 for preterm CS. (F) n=22 for vaginal, n=13 for term CS and n=13 for preterm CS.

Discussion

Over the past couple of decades, the constant rise in rates of birth by C-section has raised concern due to its positive association with an increased incidence of neurodevelopmental disorders (Glasson et al. 2004, Chudal et al. 2014, Curran et al. 2015, Brander et al. 2016, 2017, Yip et al. 2017). Yet, epidemiological studies are limited as they inherently include variable parameters that can hardly be individualized or generalized. In this study, we used a mouse model of birth by C-section under controlled conditions, aiming to determine its possible deleterious sequels at different levels. Our results suggest that birth by C-section induces transient developmental delays dependent on the gestational age at the time of delivery, but no long-term alterations. However, additional early insults might aggravate these transient sequels, leading to more persistent and deleterious consequences.

Preterm C-Section Delivery Alters Early Communicative Behavior But Does Not Lead to Long-Term Behavioral Modifications

ASD are neurodevelopmental disorders characterized by three main core symptoms: impairment in social behavior, difficulties in verbal and nonverbal communications, and a narrow range of actions carried out repetitively (WHO 2017a). Even though using animal models to study neuropsychiatric disorders (such as ASD) is challenging, behavioral tests have been widely used to evaluate pathological symptoms in

rodents (Kazdoba et al. 2016) and remain a useful aid to complement physiological studies (Del Pino, Rico, and Marin 2018). In young-adult male mice, repetitive behavior was mildly increased in mice born by C-section delivery at term but not preterm, whereas social behavior was not affected by C-section delivery, be it at term or preterm. Because social interaction tasks usually solicit exploratory and anxiety-like behavior as well as social behavior, it was also necessary to assess these parameters separately to avoid confounding the outcome of this test. As a result, we found that birth by C-section delivery did not modify locomotor and anxiety-like behaviors. However, neonatal ultrasonic vocalizations (USVs) were altered in mice born preterm by C-section compared with vaginally delivered ones. It is noteworthy that USVs are considered an early communicative behavior as well as the sign of an aversive affective state, and as such, their analysis has not only been widely applied to neurodevelopmental studies (Scattoni, Crawley, and Ricceri 2009), but has also been described as an autistic-like feature. In addition, repetitive behaviors seen in ASD are also a component of attention deficit hyperactivity disorder (ADHD), Tourette syndrome and obsessive-compulsive disorder (Rapanelli et al. 2017, Rizzo et al. 2017). Moreover, a disruption in social interactions has been associated with schizophrenia and bipolar disorder (Pappas et al. 2017) whereas an increase in locomotor activity is a parameter seen in pathologies such as schizophrenia, ADHD, or bipolar disorder (Powell and Miyakawa 2006). Altogether, our results suggest that C-section delivery does not lead to long-term behavioral alterations such as ASD, but prematurity associated to C-section delivery does affect developmental behaviors.

C-Section Delivery Does Not Impact GABA and Glutamate Activities in Juvenile Mice

An imbalance between excitation and inhibition has been suggested to underlie numerous neurodevelopmental disorders (Janik et al. 2010, Bozzi, Provenzano, and Casarosa 2018, Chiu et al. 2018), hence the need to study the effect of C-section delivery on GABAergic and glutamatergic signaling. Indeed, our lab previously showed that these two systems are affected in 2-week old pups of two different rodent models of autism with an excitatory action of GABA and an increase in activity of spontaneous glutamatergic postsynaptic currents (Tyzio et al. 2014). Yet, in the current study, mice delivered by C-section did not show any of those changes, as glutamatergic and GABAergic spontaneous activities as well as GABA polarity are similar to age-matched vaginal pups. The slightly depolarizing action of GABA observed is in agreement with our earlier determination of DF_{GABA} (Tyzio et al. 2008) and might be explained by the permeability of $GABA_A$ channels to bicarbonate ions and the NKCC1-dependant chloride equilibrium potential. In fact, in bicarbonate-free conditions or following the blockade of the chloride importer NKCC1 with its antagonist bumetanide, the previously slightly depolarizing DF_{GABA} became hyperpolarizing (Tyzio et al. 2008). Furthermore, the slight depolarization observed in all our groups is associated with inhibitory actions of GABA suggesting, as shown in earlier studies including our own, that this depolarization underlies a shunting inhibition (Monsivais, Yang, and Rubel 2000, Vida, Bartos, and Jonas 2006, Howard, Burger, and Rubel 2007, Tyzio et al. 2008, Tang et al. 2011). Altogether, these results suggest that C-section delivery at term or preterm does not lead to long-term alterations in GABAergic and glutamatergic hippocampal synaptic activities.

CA3 Pyramidal Neurons of Mice Born Preterm by C-Section are Transiently Underdeveloped

Delivery and birth are critical periods associated with major physiological changes (Jaykka and Laakso 1967, Bland et al. 1982, Ward Platt and Deshpande 2005, Tyzio et al. 2006, Hooper, Te Pas, and Kitchen 2016), yet little is known on brain operation prior to and right after birth. Rabinowicz and colleagues showed that the late gestational period in humans was accompanied by a rapid decline of neuronal cortical density which stabilizes after birth (Rabinowicz et al. 1996). C-section delivery at term or preterm might affect this sequence. Here, we show that neurons of pups delivered by C-section at term or preterm have shorter total dendritic length and fewer intersections than vaginally delivered ones. Prematurity was also an aggravating factor for the morphological development of these neurons since they present a smaller primary dendritic length and shorter ending and critical radius. These results could be due to a lower expression of the mitochondrial uncoupling protein 2 which at birth is decreased in pups born by C-section delivery and is involved in neuronal size and dendritic arbor in culture (Simon-Areces et al. 2012). Furthermore, our results are in accordance with neuronal and brain growth alterations reported in autistic individuals (Courchesne, Carper, and Akshoomoff 2003, Redcay and Courchesne 2005, Wegiel et al. 2010, Petinou and Minaidou 2017), which support the hypothesis of aberrant brain connections impeding functional brain connectivity. Animal models of ASD further support these findings as brain area-dependent morphological alterations have been previously described in such models. In the ANKRD11-deficient mouse model of KBG syndrome, which presents autistic behavior, cortical pyramidal neurons had morphological alterations which consisted in fewer number of dendrites and shorter arborization than control neurons (Ka and Kim 2018). Similar observations were done in 15 weeks old CD38 deficient mice, an ASD candidate gene (Nelissen et al. 2018). In the dentate gyrus and the visual cortex of these mice, the cell number was lower and the neurons shorter, whereas CA1 was only affected in terms of neuronal morphology, with shorter pyramidal cells (Nelissen et al. 2018). However, in contrast to our results, the morphological alterations were long-lasting

and not restricted to 1 day postnatal as here. Altogether, our results suggest that even though developmental delays are observed after being born by C-section delivery, they are not sufficient to underlie long-lasting ASD features.

Limitations of the Use of a Mouse Model and its Correspondence to Humans

Rodent models are essential to understand the fundamental mechanisms underlying the onset of neurodevelopmental disorders, but their use comes with intrinsic limitations that are important to address. In these species, as gestational length differs, so does development. The Carnegie and Theiler stages comparison suggest that a mouse *in utero* is similar to a human fetus during its first and second trimesters (Otis and Brent 1954). The third trimester of gestation in humans corresponds to the early postnatal development in rodents, leading to a human baby brain at birth being comparable to a P7 rat brain (Dobbing and Sands 1979). Even though development takes places at different ages in humans and rodents, developmental patterns are conserved across species. Indeed, the switch in sensory processing from a "bursting" mode to an adult-like "acuity" mode happens at birth in humans and before eye opening (P14-15) in rats (Colonnese et al. 2010). In the hippocampus, developmental patterns and spontaneous network-driven endogenous activities are also similar despite occurring mostly *in utero* in primates (Berger and Alvarez 1996, Khazipov et al. 2001) and humans (Kostovic et al. 1989) but shifting to *ex-utero* in rodents (Tyzio et al. 1999). Furthermore, in rodents, oxytocin receptor blockers lower the threshold of pain at birth while bumetanide and oxytocin reverse this effect (Mazzuca et al. 2011). Interestingly, human babies born by C-section also have a lower threshold of pain compared with those born vaginally (Bergqvist et al. 2009), suggesting that the analgesic effect of oxytocin at birth might be preserved across species. These commonalities between species allowed us to test the hypothesis that birth is a critical period, and that changes in the mode or time of delivery around birth could impact this important developmental milestone.

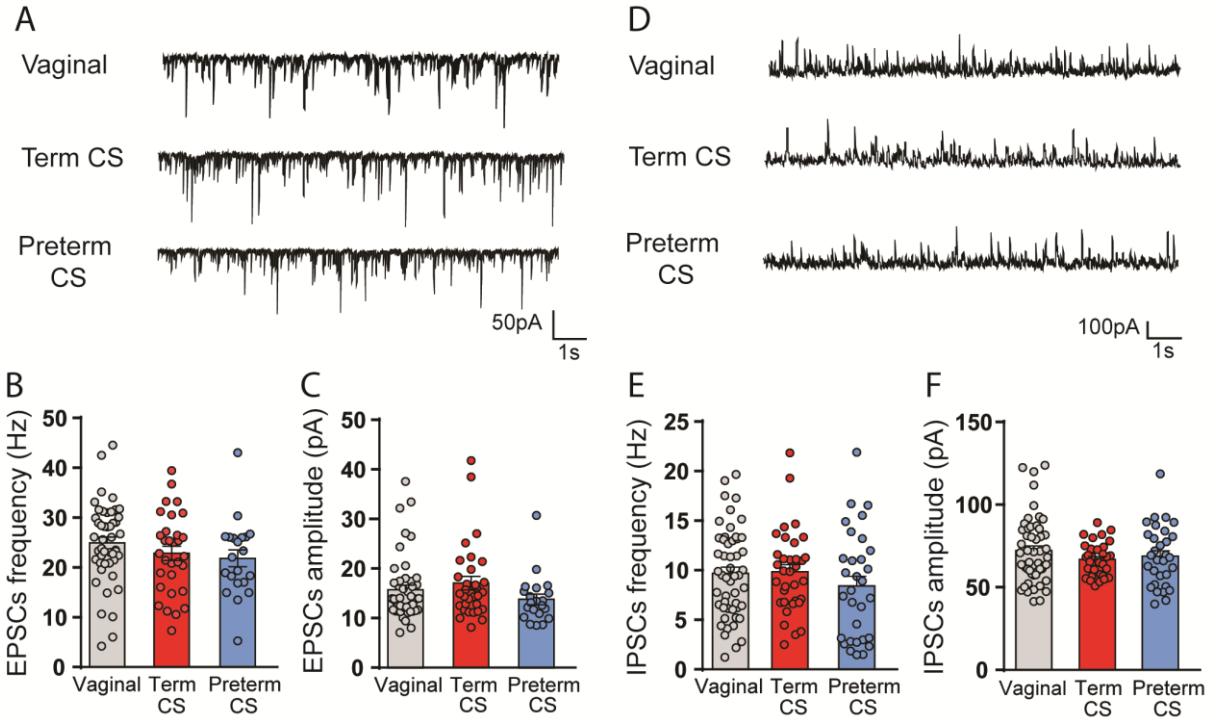

Figure 5. CS delivery at term or preterm does not affect spontaneous glutamatergic and GABAergic network activities in CA3 pyramidal neurons at P14-P15. (A) Representative traces of sEPSCs for vaginal, term CS and preterm CS pyramidal neurons (holding potential: -70 mV). Histograms of the averaged (B) frequency and (C) amplitude of sEPSCs in vaginal, term CS and preterm CS mice. (D) Representative traces of sIPSCs for vaginal, term CS and preterm CS pyramidal neurons (holding potential: +5 mV). Histograms of the averaged (E) frequency and (F) amplitude of sIPSCs in vaginal, term CS and preterm CS mice. Data are presented as mean ± SEM. (B and C) n=47 for vaginal, n=31 for term CS and n=21 for preterm CS. (E and F) n=51 for vaginal, n=34 for term CS and n=31 for preterm CS.

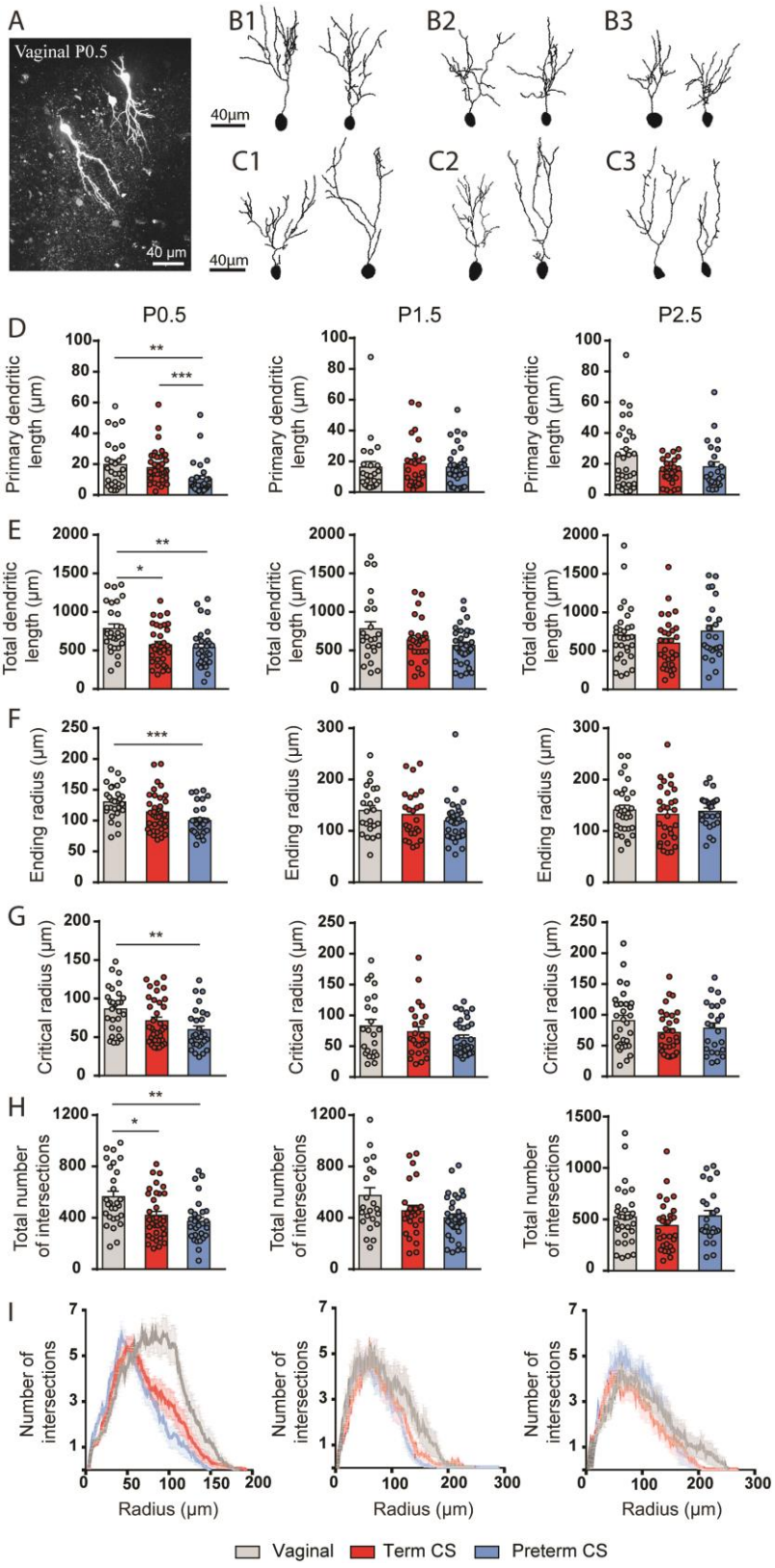

Figure 6. CA3 pyramidal cells apical morphology is transiently altered in mice born by CS delivery. (A) Image of a biocytin-filled vaginal neuron acquired with a confocal microscope. (B) Reconstruction of representative neurons at P0.5 for vaginal (B1), term CS (B2) and preterm CS (B3) mice. (C) Reconstruction of representative neurons at P1.5 for vaginal (C1), term CS (C2) and preterm CS (C3) mice. Average values in CA3 pyramidal neurons from vaginal, term CS and preterm CS mice at P0.5, P1.5 and P2.5 for (D) primary apical dendritic length (E) total apical dendritic length, (F) ending radius, (G) critical radius, (H) total number of intersections in the apical arbor and (I) sholl analysis. Data are presented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001. At

P0.5, n=28 for vaginal, n=35 for term CS and n=29 for preterm CS. At P1.5, n=23 for vaginal, n=25 for term CS and n=33 for preterm CS. At P2.5 n=30 for vaginal, n=30 for term CS and n=24 for preterm CS.

Relevance of our observations to human epidemiological studies

Epidemiological studies aimed at evaluating if C-section delivery might be associated with an increased incidence of disorders such as ASD have not yet been conclusive. They remain limited as few of them differentiate the reasons for C-section delivery such as due to an emergency (because of health risks for the mother and/or the baby) or if it was planned. In addition, only one study compared C-section across gestational ages with the probability of developing ASD (Yip et al. 2017). The authors concluded that the odds for developing ASD were similar for children born by C-section delivery between 36 and 42 weeks of gestation, but were highly variable before 36 weeks of gestation, rendering the conclusion at these ages more difficult. Furthermore, in these studies, children born by C-section delivery were neither assessed for their genetic background nor the environment their mother was exposed to during pregnancy, even though ASD has been associated with those factors. For these reasons, as of now, it is impossible to know if the increased incidence of ASD in children born by C-section delivery is due to one or more external factor(s) or to the surgical act by itself. Our study sheds light on this issue by showing that C-section delivery at term or preterm induces transient developmental delays but does not lead to long-term consequences nor to autistic-like features *per se*. Interestingly, we also show that prematurity could be an aggravating factor, and an earlier prematurity might have led to permanent alterations in our experimental conditions. Unfortunately, we could not test this possibility as we failed to obtain viable pups born earlier than E17.75. This might be explained by the lung immaturity at earlier gestational ages in mice. In fact, before E17.4, the lymphatic network in lung tissue is not well developed and the surfactant synthesis and secretion, which allow gas exchange, are immature (Warburton et al. 2010). Finally, we cannot exclude the possibility that a double hit combining C-section delivery with an early or postnatal insult might lead to the increased incidence of ASD observed in some epidemiological studies (Glasson et al. 2004, Al-Ansari and Ahmed 2013, Curran et al. 2015, Yip et al. 2017). Indeed, these transient developmental alterations seen in mice born by C-section delivery might be aggravated by an early in utero, neonatal or postnatal insult. In this scheme, future studies should focus on the hypothesis that delivery is a critical period whose alteration may play an aggravating role to a secondary insult.

Supplementary Material

Supplementary materials is available at Cerebral Cortex online.

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Notes

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Supplementary Material

Term or Preterm Cesarean Section Delivery Does Not Lead to Long-term Detrimental Consequences in Mice

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Supplementary Table 1. Social behavior in young-adult male mice is not affected by C-section delivery. (1) Numbers correspond to Figure 1B. (2) Numbers correspond to Figure 1D. Statistics are presented by the Mann-Whitney test.

(1) Figure 1B	Time spent in empty chamber (s), mean ± SEM	Time spent with mouse (s), mean ± SEM	Statistics
Vaginal (n=33)	181 ± 9.91	314 ± 12.25	**p<0.0001**
Term CS (n=23)	189 ± 12.93	299.9 ± 15.47	**p<0.0001**
Preterm CS (n=19)	208.3 ± 10.66	271.2 ± 14.88	**p=0.0027**
(2) Figure 1D	Time spent with familiar mouse (s), mean ± SEM	Time spent with novel mouse (s), mean ± SEM	Statistics
Vaginal (n=33)	225.6 ± 9.87	245.2 ± 9.39	p=0.1782.
Term CS (n=23)	219 ± 13.15	247.1 ± 13.63	p=0.0687
Preterm CS (n=19)	228.3 ± 10.09	233.9 ± 10.79	p=0.3211

Supplementary Table 2. Stereotypic behaviors are mildly increased in mice born at term by C-section, but no other alterations can be seen between vaginal, term C-section and preterm C-section mice. (1) Numbers correspond to Figure 1E. (2) Numbers correspond to Figure 1F. (3) Numbers correspond to Figure 1G. Statistics are presented by the Kruskal-Wallis with Dunn's post-hoc test.

	Vaginal (n=33)	Term CS (n=22)	Preterm CS (n=17)	Statistics
(1) For Figure 1E				
Grooming time (s), mean ± SEM	114.9 ± 10.95	163.7 ± 13.39	121.8 ± 15.99	**Vaginal *vs.* term CS, p=0.0219** Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p=0.0693
(2) For Figure 1F				
Number of events, mean ± SEM	8.52 ± 0.88	7.82 ± 0.72	9.47 ± 1.42	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p>0.9999
(3) For Figure 1G				
Latency to start grooming (s), mean ± SEM	72.55 ± 12.1	75.77 ± 16.69	53.12 ± 17.22	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p=0.8512 Term CS *vs.* preterm CS, p>0.9999

Supplementary Table 3. Anxiety-like behavior is not affected by C-section delivery. (1) Numbers correspond to Figure 2B. (2) Numbers correspond to Figure 2C. (3) Numbers correspond to Figure 2D. Statistics are presented by the Kruskal-Wallis with Dunn's post-hoc test.

	Vaginal (n=33)	Term CS (n=23)	Preterm CS (n=19)	Statistics
(1) For Figure 2B				
Time spent in the center (s), mean ± SEM	45.71 ± 3.6	49.29 ± 3.77	39.04 ± 3.28	Vaginal *vs.* term CS, p=0.8516 Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p=0.1988
(2) For Figure 2C				
Number of entries in center, mean ± SEM	47.64 ± 2.5	46.26 ± 2.88	42.84 ± 3.34	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p=0.7350 Term CS *vs.* preterm CS, p>0.9999
(3) For Figure 2D				
Latency to enter center (s), mean ± SEM	12.76 ± 2.61	16.92 ± 4.29	13.64 ± 3.42	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p>0.9999

Supplementary Table 4. Locomotor activity is not different in term and preterm C-section compared to vaginal mice. (1) Numbers correspond to Figure 2E. (2) Numbers correspond to Figure 2F. (3) Numbers correspond to Figure 2G. Statistics are presented by Kruskal-Wallis with Dunn's post-hoc test.

	Vaginal (n=33)	Term CS (n=23)	Preterm CS (n=19)	Statistics
(1) For Figure 2E				
Velocity (cm/s), mean ± SEM	7.18 ± 0.3	6.55 ± 0.2	6.49 ± 0.37	Vaginal *vs.* term CS, p=0.6458 Vaginal *vs.* preterm CS, p=0.3576 Term CS *vs.* preterm CS, p>0.9999
(2) For Figure 2F				
Distance traveled (cm), mean ± SEM	4301 ± 177.5	3919 ± 120.5	3893 ± 220.5	Vaginal *vs.* term CS, p=0.6053 Vaginal *vs.* preterm CS, p=0.3474 Term CS *vs.* preterm CS, p>0.9999
(3) For Figure 2G				
Moving time (s), mean ± SEM	440.7 ± 8.5	419.2 ± 9.02	408.6 ± 10.44	Vaginal *vs.* term CS, p=0.5522 Vaginal *vs.* preterm CS, p=0.0898 Term CS *vs.* preterm CS, p>0.9999

Supplementary Table 5. Pups born preterm by C-section delivery present early communicative alterations compared to vaginal born pups. (1) Numbers correspond to Figure 3A. (B) Numbers correspond to Figure 3B. (C) Numbers correspond to Figure 3C. Statistics are presented by Kruskal-Wallis with Dunn's post-hoc test.

	Vaginal (n=55)	Term CS (n=20)	Preterm CS (n=35)	Statistics
(1) For Figure 3A				
Number of calls, mean ± SEM	89.05 ± 14.14	143 ± 28.96	206.7 ± 29.25	Vaginal *vs.* term CS, p=0.1323 **Vaginal *vs.* preterm CS, p=0.0013** Term CS *vs.* preterm CS, p>0.9999
(2) For Figure 3B				
Total calling time (s), mean ± SEM	3.24 ± 0.55	5.06 ± 0.99	8.17 ± 1.27	Vaginal *vs.* term CS, p=0.1703 **Vaginal *vs.* preterm CS, p=0.0011** Term CS *vs.* preterm CS, p=0.9833
(2) For Figure 3C				
Call duration (ms), mean ± SEM	32.44 ± 1.16	34.48 ± 1.24	36.15 ± 1.23	Vaginal *vs.* term CS, p=0.8831 Vaginal *vs.* preterm CS, p=0.1469 Term CS *vs.* preterm CS, p>0.9999

Supplementary Table 6. The driving force of $GABA_A$ receptors in CA3 pyramidal neurons is similar for vaginal, term and preterm C-section mice. Statistics are presented by two tailed *t* test with numbers corresponding to Figure 4A.

	Vaginal (n=31)	Term CS (n=19)	Preterm CS (n=15)	Statistics
DF_{GABA} (mV), mean ± SEM	4.1 ± 3.6	4.7 ± 5.8	2.6 ± 2.9	Vaginal *vs.* term CS, p=0.226 Vaginal *vs.* preterm CS, p=0.357

Supplementary Table 7. Application of the $GABA_A$ receptor agonist isoguvacine (10µM) decreases the frequency of spikes (% of control) in extracellular field recordings of hippocampal slices. Statistics are presented by the repeated measures one-way ANOVA with Dunnett's multiple comparisons post-hoc test with numbers corresponding to Figure 4F.

	Isoguvacine (%), mean ± SEM	Wash-out (%), mean ± SEM	Isoguvacine *vs.* control	Wash-out *vs.* control
Vaginal (n=22)	78.89 ± 2.71	99.87 ± 2.57	**p=0.0001**	p=0.9982
Term CS (n=13)	73.92 ± 5.47	93.88 ± 2.41	**p=0.0009**	p=0.0907
Preterm CS (n=13)	79.55 ± 3.23	97.51 ± 2.35	**p=0.0001**	p=0.4867

Supplementary Table 8. Spontaneous activities of hippocampal CA3 pyramidal networks are not different in vaginal, term and preterm C-section mice. (1) Numbers correspond to Figure 5B and 5C. (2) Numbers correspond to Figure 5E and 5F. For Figure 5B and 5E, statistics are presented by ANOVA with Tukey's post-hoc test. For Figure 5C and 5F, statistics are presented by Kruskal-Wallis with Dunn's post-hoc test.

	Vaginal	Term CS	Preterm CS	Statistics
(1) For Figure 5B and 5C				
sEPSCs frequency (Hz), mean ± SEM	24.93 ± 1.18 (n=47)	22.88 ± 1.45 (n=31)	21.87 ± 1.68 (n=21)	Vaginal *vs.* term CS, p=0.5107 Vaginal *vs.* preterm CS, p=0.3169 Term CS *vs.* preterm CS, p=0.8973
sEPSCs amplitude (pA), mean ± SEM	15.76 ± 0.94 (n=47)	17.01 ± 1.37 (n=31)	13.85 ± 1.06 (n=21)	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p=0.7670 Term CS *vs.* preterm CS, p=0.2537
(2) For Figure 5E and 5F				
sIPSCs frequency (Hz), mean ± SEM	9.68 ± 0.63 (n=51)	9.84 ± 0.72 (n=34)	8.39 ± 0.97 (n=31)	Vaginal *vs.* term CS, p=0.9862 Vaginal *vs.* preterm CS, p=0.4466 Term CS *vs.* preterm CS, p=0.4230
sIPSCs amplitude (pA), mean ± SEM	72.02 ± 2.81 (n=51)	66.57 ± 1.69 (n=34)	68.82 ± 3.26 (n=31)	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p>0.9999

Supplementary Table 9. Morphological characteristics of term CS, preterm CS and vaginal CA3 pyramidal neurons at P0.5. Statistics are presented by Kruskal-Wallis with Dunn's post-hoc test. Data are presented as mean ± SEM.

	Vaginal (n=28)	Term CS (n=35)	Preterm CS (n=29)	Statistics
Primary dendritic length (µm)	19.72 ± 2.88	19.04 ± 1.92	10.39 ± 2.09	Vaginal *vs.* term CS, p>0.9999 **Vaginal *vs.* preterm CS, p=0.0054** **Term CS *vs.* preterm CS, p=0.0003**
Mean dendritic length (µm)	19.6 ± 1.02	19.89 ± 1.11	17.53 ± 0.89	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p=0.4129 Term CS *vs.* preterm CS, p=0.2990
Total dendritic length (µm)	784.6 ± 58.83	573 ± 42.04	537.2 ± 49.43	**Vaginal *vs.* term CS, p=0.0218** **Vaginal *vs.* preterm CS, p=0.004** Term CS *vs.* preterm CS, p>0.9999
Ending radius (µm)	130.4 ± 5.11	113.8 ± 5.37	99.9 ± 4.73	Vaginal *vs.* term CS, p=0.0671 **Vaginal *vs.* preterm CS, p=0.0004** Term CS *vs.* preterm CS, p=0.2602
Critical radius (µm)	86.61 ± 5.63	71.08 ± 4.94	59.51 ± 4.90	Vaginal *vs.* term CS, p=0.1629 **Vaginal *vs.* preterm CS, p=0.0022** Term CS *vs.* preterm CS, p=0.3118
Nb of intersections	563.1 ± 43.05	419.9 ± 30.52	370.8 ± 31.21	**Vaginal *vs.* term CS, p=0.0265** **Vaginal *vs.* preterm CS, p=0.0017** Term CS *vs.* preterm CS, p=0.9642
Mean number of intersections per dendrite	4.479 ± 0.26	3.892 ± 0.22	3.906 ± 0.25	Vaginal *vs.* term CS, p=0.2661 Vaginal *vs.* preterm CS, p=0.4125 Term CS *vs.* preterm CS, p>0.9999

Supplementary Table 10. Morphological characteristics of term CS, preterm CS and vaginal CA3 pyramidal neurons at P1.5. Statistics are presented by Kruskal-Wallis with Dunn's post-hoc test. Data are presented as mean ± SEM.

	Vaginal (n=23)	Term CS (n=25)	Preterm CS (n=33)	Statistics
Primary dendritic length (µm)	16.1 ± 3.77	18.42 ± 3.18	16.23 ± 2.22	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p>0.9999
Mean dendritic length (µm)	22.47 ± 1.87	22.37 ± 1.95	18.714 ± 1.13	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p=0.1956 Term CS *vs.* preterm CS, p=0.5040
Total dendritic length (µm)	780.3 ± 92.15	634.1 ± 57.82	566.2 ± 42.19	Vaginal *vs.* term CS, p=0.9753 Vaginal *vs.* preterm CS, p=0.2427 Term CS *vs.* preterm CS, p>0.9999
Ending radius (µm)	139.6 ± 9.93	131.8 ± 9.62	119.4 ± 7.34	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p=0.2887 Term CS *vs.* preterm CS, p>0.9999
Critical radius (µm)	82.85 ± 10.58	73.14 ± 8.48	63.42 ± 4.89	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p=0.8911 Term CS *vs.* preterm CS, p>0.9999
Nb of intersections	573.8 ± 62.05	454.9 ± 42.67	401.5 ± 29.32	Vaginal *vs.* term CS, p=0.6857 Vaginal *vs.* preterm CS, p=0.0794 Term CS *vs.* preterm CS, p>0.9999
Mean number of intersections per dendrite	4.44 ± 0.39	3.713 ± 0.27	3.62 ± 0.23	Vaginal *vs.* term CS, p=0.5534 Vaginal *vs.* preterm CS, p=0.2565 Term CS *vs.* preterm CS, p>0.9999

Supplementary Table 11. Morphological characteristics of term CS, preterm CS and vaginal CA3 pyramidal neurons at P2.5. Statistics are presented by Kruskal-Wallis with Dunn's post-hoc test. Data are presented as mean ± SEM.

	Vaginal (n=30)	Term CS (n=30)	Preterm CS (n=24)	Statistics
Primary dendritic length (µm)	25.69 ± 3.93	15.33 ± 1.45	18.06 ± 3.16	Vaginal *vs.* term CS, p=0.6008 Vaginal *vs.* preterm CS, p=0.5965 Term CS *vs.* preterm CS, p>0.9999
Mean dendritic length (µm)	20.62 ± 1.66	16.09 ± 0.97	21.85 ± 1.87	Vaginal *vs.* term CS, p=0.0588 Vaginal *vs.* preterm CS, p>0.9999 **Term CS *vs.* preterm CS, p=0.0188**
Total dendritic length (µm)	705.6 ± 69.18	601.6 ± 60.37	755.8 ± 78.56	Vaginal *vs.* term CS, p=0.7328 Vaginal *vs.* preterm CS, p>0.999 Term CS *vs.* preterm CS, p=0.4210
Ending radius (µm)	140.9 ± 9.11	132 ± 9.83	138.1 ± 6.87	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p>0.9999
Critical radius (µm)	90.61 ± 8.65	71.13 ± 6.56	78.33 ± 8.39	Vaginal *vs.* term CS, p=0.2993 Vaginal *vs.* preterm CS, p=0.8858 Term CS *vs.* preterm CS, p>0.9999
Nb of intersections	522.9 ± 52.06	439 ± 43.63	533.1 ± 54.29	Vaginal *vs.* term CS, p=0.5521 Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p=0.5644
Mean number of intersections per dendrite	3.783 ± 0.23	3.493 ± 0.21	4.016 ± 0.36	Vaginal *vs.* term CS, p>0.9999 Vaginal *vs.* preterm CS, p>0.9999 Term CS *vs.* preterm CS, p>0.9999

DISCUSSION

This thesis aimed to investigate if C-section delivery is associated with increased risks of neurodevelopmental disorders such as ASD, as seen in epidemiological studies[252,448]. Our research shows that C-section delivery, either at term or preterm, does not increase the risk of developing ASD in mice. Indeed, birth by C-section only induces early transient developmental delays that are dependent on the gestational age at the time of delivery.

Behavioral evaluation of ASD in mice born by C-section.

ASD are neurodevelopmental disorders diagnosed in patients presenting three core symptoms: persistent deficits in social communication and social interactions, deficits in nonverbal communicative behaviors, and restrictive and stereotypic behaviors[256]. To evaluate ASD in animal models, behavioral tests have been used to characterize if animals were autistic-like. Amongst the many behavioral tests developed in rodents, three have been widely used to test the three core symptoms of ASD: (1) the three-chamber social test, (2) the grooming test, and (3) ultrasonic vocalizations (USVs) either during the neonatal period or later[449].

Because grooming and social behaviors could be influenced by anxiety, assessing the later parameter alone was necessary to avoid confounding the outcome of each test. Our results show that in young-adult male mice, C-section delivery does not induce changes in anxiety-like nor locomotor behaviors. Regarding ASD-related behaviors, young-adult male mice delivered by C-section at term present a mild increase in repetitive behaviors, whereas social behaviors are not affected by C-section delivery (be it at term or preterm). However, during the neonatal period, USVs are increased in mice born preterm by C-section delivery compared to vaginally delivered ones. Hence, our results suggest that C-section delivery does not lead to long-term ASD behavioral alterations, but prematurity associated to C-section delivery does affect neonatal behaviors.

Interestingly, the behaviors we tested are not exclusive to ASD and might give us insights on other neurodevelopmental disorders. Indeed, neonatal USVs are considered an early communicative behavior as well as the sign of an aversive state, and as such, their analysis has not only been applied to ASD, but also to other neurodevelopmental disorders[450]. Repetitive behaviors, in addition to ASD, are also a component of ADHD, TS, and OCD[451,452], while disruption of social interactions belongs to the symptomatology of neurodevelopmental

disorders such as schizophrenia and BD[453]. In addition, locomotor activity is enhanced in animal models for schizophrenia, ADHD, and BD[454].

It is noteworthy that C-section delivery has been reported to increase the risk of developing all these pathologies[229,231,232,235,236]. Thus, behavioral tests used to characterize ASD, even though insufficient to adequately assess other pathologies, allow us to speculate that C-section delivery might not lead to the development of any of these diseases. To confirm this hypothesis, additional behavioral tests will need to be evaluated and include the elevated plus-maze or light-dark exploration tests for anxiety[455], and the rotarod and gait analysis tests for motor coordination and balance[456].

In addition, our observation that preterm C-section delivery induces an alteration of neonatal USVs could be a sign that other developmental behaviors could be at risk. To test this hypothesis, we could assess developmental milestones/reflexes in mice delivered by C-section. At birth, pups' movements are uncoordinated, tactile sensitivity is not mature, and ear canals and eyes are closed. These processes develop during the postnatal period and can be used to evaluate if C-section delivery induces developmental delays. Milestones are divided into three categories: the developmental reflexes, the development of locomotor behaviors, and physical landmarks[457]. Our preliminary data shows that mice delivered by C-section are underweight during development, and preterm C-section delivered mice open their eyes later than the two other groups (Figure 16; Chiesa et al., unpublished data). Hence, assessing other developmental milestones might be valuable to understand which processes might be affected or delayed by C-section delivery.

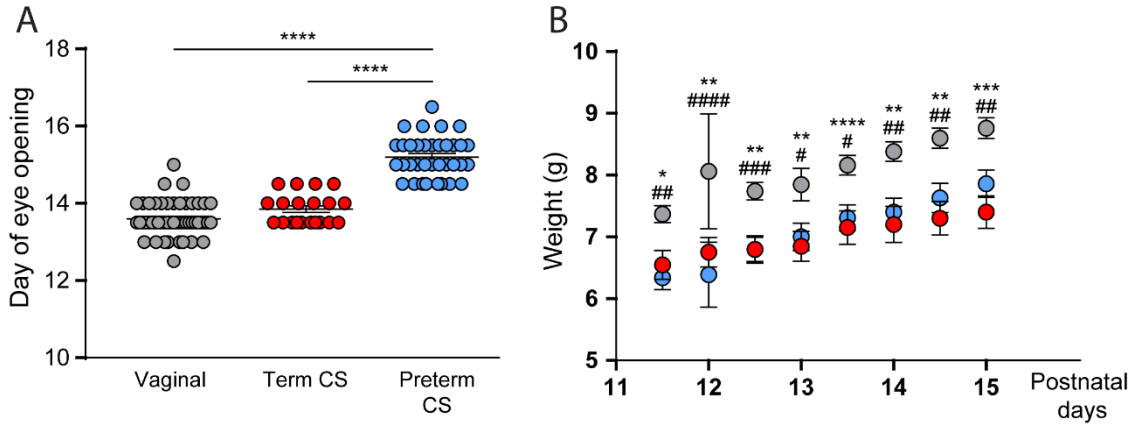

Figure 16. Developmental milestones in mice born by C-section delivery at term or preterm. (A) Postnatal day of eye opening. (B) Weight during the second postnatal week. * vaginal versus term CS mice, # vaginal versus preterm CS mice. Data are presented as mean ± SEM. (A and B) n=50 for vaginal (grey), n=20 for term CS (red), and n=35 for preterm CS (blue). Data are presented as mean ± SEM, $^{\#}$ or *P < 0.05, $^{\#\#}$ or **P < 0.01, $^{\#\#\#}$ or ***P < 0.001, $^{\#\#\#\#}$ or ****P < 0.0001. Data are analyzed by the Kruskal-Wallis with Dunn's post hoc test.

Assessment of the balance between excitation and inhibition during development.

In wild-type mice and rats, birth is associated with a transient oxytocin-mediated hyperpolarizing shift[126]. Moreover, GABA shifts from excitatory to inhibitory during the second postnatal week, even though the polarity of GABA remains slightly depolarizing[127,458]. This observation might be explained by the permeability of $GABA_A$ channels to HCO_3^- in addition to Cl^-, and the NKCC1-dependent chloride equilibrium potential. Indeed, in HCO_3^--free conditions or following the blockade of NKCC1 with bumetanide, the polarity of GABA becomes hyperpolarizing[458]. Moreover, this association between slightly depolarizing but inhibitory actions of GABA might underlie a shunting inhibition, as seen in other studies[458–460].

An imbalance between excitation and inhibition has been suggested to underlie numerous neurodevelopmental disorders[461–463], hence the need to study the effect of C-section delivery on GABAergic and glutamatergic signaling in the brain. Indeed, as described in the introduction of this manuscript, alterations in glutamate and GABA systems have been associated with ASD. Moreover, our lab's previous results showed that in hippocampal CA3 pyramidal neurons of two rodent models of autism the balance is shifted in favor of an excitatory and hyperactive network. Indeed, the transient oxytocin-mediated shift is abolished at birth, GABA actions are excitatory and the frequency of spontaneous glutamatergic postsynaptic currents is increased during the second postnatal week, with an enhanced power of network oscillations *in vivo* (power in the α, β, θ and γ frequency bands)[127].

We found that in mice delivered by C-section at term or preterm, hippocampal glutamatergic and GABAergic spontaneous activities during the second postnatal week were similar to age-matched vaginal pups. In addition, the GABA polarity was slightly depolarizing and associated with an inhibitory action of GABA. Hence, C-section delivery does not induce an imbalance between excitation and inhibition as seen in rodent models of autism.

Neuronal growth following C-section delivery.

Delivery and birth are critical periods associated with major physiological changes (as described in the introduction), yet little is known on brain operation prior to and right after birth.

In humans, cortical thickness increases throughout pregnancy and early postnatal life, while neuronal density decreases starting from 20 weeks of gestation and stabilizes after birth[464]. In ASD patients, brain growth is decreased during the first postnatal years, and increased after 6 years of life compared to non-autistic patients[465,466] (see Figure 6, p.46). In addition, abnormal

neuronal migration and atypical brain morphology areas are reported in autistic individuals[467,468]. These observations support the hypothesis of aberrant brain connections impeding functional brain connectivity in ASD.

Animal models of ASD further support these findings as brain area-dependent morphological alterations have been previously described in such models. In the ANKRD11-deficient mouse model of KBG syndrome (which includes ASD-related symptoms), cortical pyramidal neurons from P14 to P42 present morphological alterations consisting in fewer number of dendrites and shorter arborization than control neurons[469]. Similar observations were done in 15 weeks old CD38 deficient mice, an ASD candidate gene[470]. In the dentate gyrus and visual cortex of these mice, the cell number was lower and the neurons shorter, whereas CA1 was only affected in terms of neuronal morphology, with shorter pyramidal cells[470]. Thus, animal models of ASD present morphological alterations that are long-lasting. For these reasons, we assessed CA3 pyramidal neurons morphology at birth and shortly after to examine if neuronal growth was altered in C-section delivered mice, and if changes were persistent, as seen in animal models of ASD.

We observed that, in mice delivered by C-section delivery at term or preterm compared to vaginal-delivered ones, CA3 pyramidal neurons have shorter total dendritic length and fewer intersections at P0.5 (Figure 17A). Moreover, prematurity acts as an aggravating factor for the morphological development of these neurons since they present a smaller primary dendritic length and shorter ending and critical radius (Figure 17A). However, contrary to what is observed in animal models of ASD, the morphological alterations are restricted to one day postnatal for neurons of mice born by C-section delivery.

Our findings might be associated to a lower expression of the mitochondrial uncoupling protein 2 which at birth is decreased in pups born by C-section delivery and is involved in neuronal size and dendritic arbor in culture[471]. Lower expression of neurotrophins might also be linked to the underdevelopment of CA3 pyramidal neurons at birth in mice born by C-section delivery. Neurotrophins are necessary for neuronal differentiation that is required for the proper formation of synaptic connections between neurons[472]. BDNF, neurotrophin-3, neurotrophin-4, and nerve growth factor have been shown to regulate the dendritic growth of pyramidal neurons in the visual cortex, with each neurotrophin preferentially promoting the neuronal growth of specific cortical layers[473]. In addition, BDNF concentrations increase throughout gestation in humans[474], transiently decline around birth, and recover during the first postpartum week[475]. Nonetheless, in preterm infants BDNF levels are lower in the umbilical cord, at 1 day

postpartum, and 4 days postpartum than in full-term infants (Figure 17B)[475]. Moreover, contrary to full-term infants where BDNF levels do not vary at any of these stages, BNDF levels significantly increase between birth and 1 day postpartum and decrease between postpartum day 1 and 4 in premature babies[475].

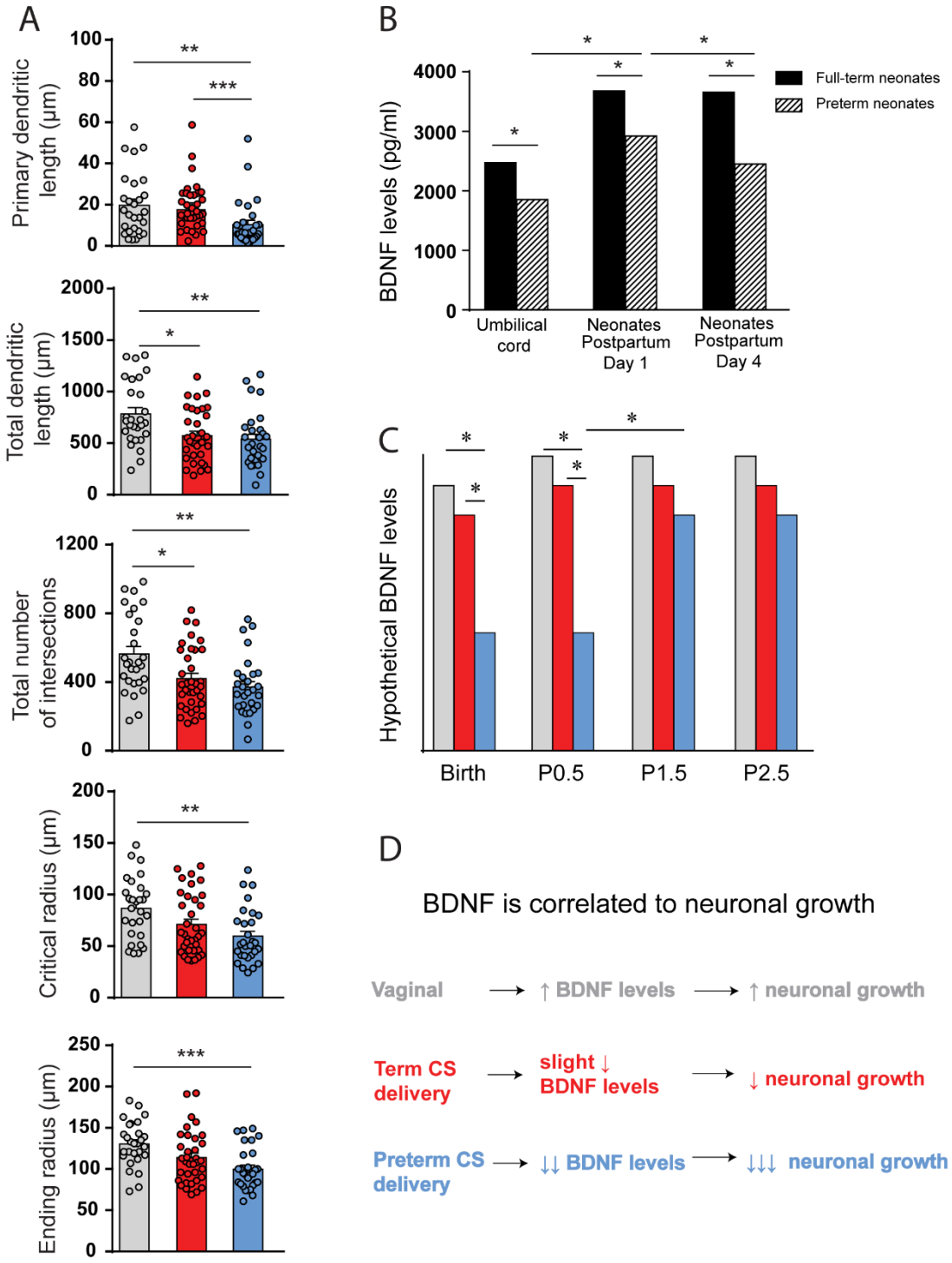

Figure 17. The BDNF hypothesis. (A) Average values in CA3 pyramidal neurons from vaginal (grey), term CS (red) and preterm CS (blue) mice at P0.5. (B) BDNF circulating levels (pg/ml) in full-term and preterm neonates. Adapted from "Perinatal changes of brain-derived neurotrophic factor in pre- and fullterm neonates", by A. Malamitsi-Puchner et al., 2004, Early Human Development, 76(2004): 17-22. (C) Perinatal hypothetical BDNF hippocampal levels after C-section delivery at term or preterm. (D) Hypothetical link between C-section delivery, BDNF, and neuronal growth in the perinatal period.

Based on these results, perinatal BDNF levels might be correlated to the neuronal growth observed in our mice delivered by C-section. Indeed, we could expect that BDNF levels at birth are slightly lower in term C-section mice compared to vaginal mice, thus explaining the minor CA3 pyramidal neurons morphological changes observed. In our preterm C-section mice, we could anticipate that BDNF levels are much lower at birth than for term C-section and vaginal-delivered mice and correlated with underdeveloped CA3 pyramidal neurons. BDNF levels might also strikingly increase between P0.5 and P1.5 (as in humans) and explain why these neurons grow remarkably faster during this period than neurons of vaginal-born mice. To test this hypothesis, we will need to dose BDNF levels at birth to see if levels are lower in the preterm C-section group than for term C-section and vaginal groups, and at P1.5 to see if in the preterm C-section group, BDNF levels strikingly increase while they could remain stable for the two other groups (Figure 17C). If our hypothesis is right, we could further correlate alterations in BDNF levels with neuronal growth by treating preterm C-section mice at birth (P0) with BDNF and comparing preterm-treated neurons to vaginal and term C-section neurons at P0.5. If neurons of these three groups are similar at P0.5, we could conclude that perinatal growth in CA3 pyramidal neurons is regulated by BDNF. Moreover, morphological alterations are worse in mice born preterm by C-section delivery than at term, suggesting that the combination of C-section delivery and low BDNF levels might play an aggravating role compared to C-section delivery alone (Figure 17D).

The anesthetics complication.

During labor and vaginal delivery, the human mother releases catecholamines, oxytocin, prostaglandins, and cortisol among other hormones. However, since birth by C-section is highly variable in terms of time of delivery, type of C-section (emergency versus planned), presence or not of labor, as well as in the use of anesthetics and/or other medications, it is hard to determine which hormones are secreted by human mothers during a C-section. In addition, in humans, C-section delivery is done under either regional anesthesia (e.g. epidural anesthesia) or general anesthesia. And, preclinical studies have shown that the use of anesthetics interfere with neurotransmitters signaling[476–478].

General anesthesia produces extensive neuronal changes in the CNS by enhancing inhibitory and reducing excitatory neurotransmission[476]. Furthermore, anesthetics bind to different $GABA_A$ receptor subunits, increasing the complexity of anesthetic actions on the GABAergic system[476]. Preclinical studies have shown that long-term consequences engendered by anesthetics were dependent on the type of anesthetics used. Indeed, exposition during 6 hours

to sevoflurane increased whole brain apoptotic neurodegeneration, and impairment in social interactions in 8 weeks old mice[479]. Mice at P7 also had increased whole brain cellular degeneration and apoptosis after a 6-hours exposition to isoflurane. However, neuronal density and behavior at adulthood were similar for neonatal pups exposed to anesthesia and those exposed to room air[480]. Thus, anesthetics used during general anesthesia must be carefully chosen to avoid long-term consequences.

Epidural anesthesia also interferes with neuronal transmission in numerous ways such as inhibiting glutamatergic ionotropic receptors in the spinal cord[481]. However, as of today, the use of epidural anesthesia has no known long-term disadvantages. In fact, to our knowledge, no studies (either clinical or preclinical) have investigated the long-term effects of epidural anesthesia during delivery (either vaginal or C-section) on the infant's brain development.

Since the use of anesthetics varies in humans, we decided to avoid their utilization in our study. We thought cervical dislocation would be the most adequate procedure enabling us to evaluate the effect of C-section as mode of delivery and its possible long-term effects. Indeed, keeping in mind that birth is a critical period, our overall goal was to evaluate the effect of C-section delivery as a modification during this critical period, and not necessarily the direct relationship between C-section delivery in humans and rodents, which will undoubtedly need the use of anesthetics.

The hormone hypothesis.

Our lab's previous data showed that oxytocin plays an important role for neuronal development at birth. Indeed, the blockade of oxytocin receptors by its antagonists (atosiban and SSR1267689) at birth abolished the transient GABA hyperpolarizing shift (Figure 18A), decreased the threshold for pain, and led to long-term physiological and behavioral consequences in rodents[125–127]. These effects seem restricted to birth since the blockade of oxytocin receptors did not modify the threshold for pain in rat pups at P2[125]. In addition, oxytocin administration and blockade of NKCC1 by bumetanide increased the threshold for pain at birth and P2. Hence, all these results suggest that oxytocin at birth might modulate intracellular chloride concentrations (Figure 18B). In fact, oxytocin has been shown to regulate KCC2 cell surface expression[482]. Binding of oxytocin to its receptor activates G_q protein which, in turn, activates PKC. PKC phosphorylates serine 940 within the C-terminal cytoplasmic domain of KCC2[482]. This phosphorylation increases the cell surface stability of KCC2 by decreasing its internalization rate, and is associated with an increased rate of ion transport by

KCC2. Higher expression of KCC2 at the cell surface induces an increase in chloride extrusion, lower intracellular chloride concentrations, hence resulting in a transient hyperpolarization of GABA when it binds to $GABA_A$ receptors.

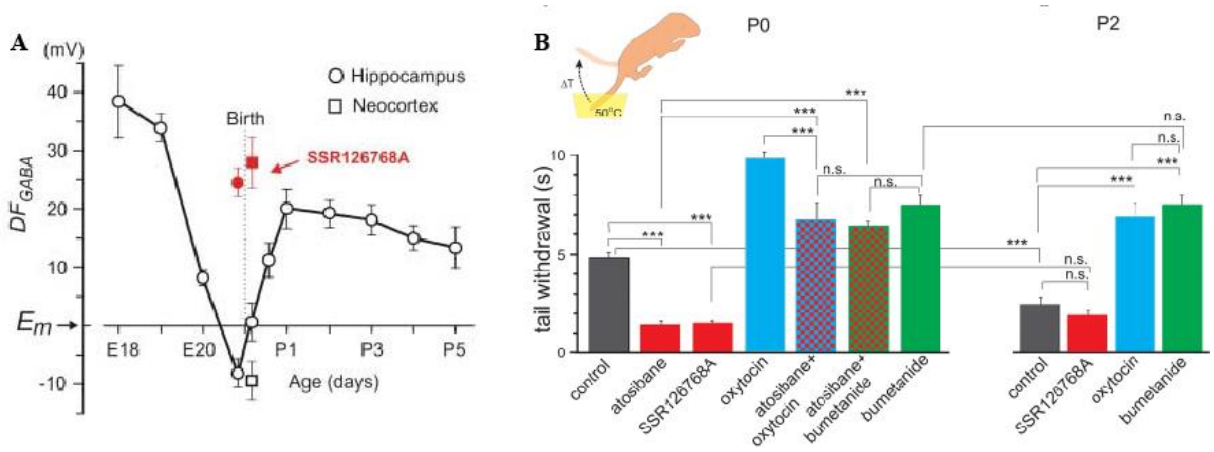

Figure 18. Neurological effects of oxytocin at birth. (A) Summary plot of the age dependence of DF_{GABA} inferred from single $GABA_A$ channels recordings. Red indicates pretreatment with the oxytocin receptor antagonist SSR1267689. Reprinted from "Maternal oxytocin triggers a transient inhibitory switch in GABA signaling in the fetal brain during delivery", by R. Tyzio et al., 2006, Science, 314(5806): 1788-1792. (B) Tail-flick pain response in the newborn rat. Reprinted from "Newborn analgesia mediated by oxytocin during delivery", by M. Mazzuca et al., 2011, Frontiers in neuroscience, 5:3.

Interestingly, human babies born at term by elective C-section have a lower threshold for pain at birth[120], suggesting that oxytocin or oxytocin receptor expressions might be altered when born by C-section delivery. However, this hypothesis is extremely hard to tackle in humans and subprimate species. Indeed, discrepancies are observed between species because, maternal and fetal oxytocin levels increase during late gestation in subprimate species, while fetal levels alone seem to increase in humans. Moreover, dosing oxytocin in the fetus could be inefficient since pups receive maternal oxytocin that is contained in breast milk. In addition, to our knowledge, no studies of the oxytocinergic system have been done during the neonatal period, thus we do not know if magnocellular neurons secreting oxytocin are active and fire properly at birth. Indeed, studies report that oxytocin depolarizes supraoptic magnocellular neurons, and that this depolarization is associated with tonic activity and oxytocin release during the second postnatal week and onwards in rats[483]. Furthermore, the dendritic arbor of these neurons is increased by oxytocin and slightly decreased by oxytocin receptors antagonist between P3 and P6, indicating that receptors are functional at these ages[484]. However, magnocellular neurons are not depolarized in response to oxytocin before P4, and this might be correlated to a higher threshold of action potentials and resting membrane potential in neurons prior to P5-P6 compared to P9-P10 and later[485]. These studies show that the oxytocinergic system might already be active during the first postnatal week, but not in the first few days after birth. Hence,

even though interesting, the assessment of oxytocin and oxytocin receptors in C-section delivery, at term or preterm, and compared to vaginal delivery might not necessarily allow us to reach straightforward conclusions.

Nonetheless, blockade of oxytocin receptors in mice one day prior to birth has been associated with depolarizing and excitatory actions of GABA at birth and during the second postnatal week[127]. Similar observations were also done in rat and mouse models of autism[127]. Hence, we could have expected that, if C-section delivery is associated with an alteration of oxytocin and/or oxytocin receptors, a depolarizing action of GABA at birth and during the second postnatal week would be seen. Yet, our results show that, similar to vaginal-born mice, GABA at P14-P15 is slightly depolarizing and associated with an inhibitory activity. We did not record DF_{GABA} at birth, but our lab's previous results indicate that GABA is depolarizing at E18 in mice[127]. Thus, we believe GABA to be depolarizing in mice delivered by C-section at E17.75 (preterm). However, the question for mice born at E18.75 (term) is more complicated. Indeed, GABA is transiently hyperpolarizing at birth, with this shift peaking right before birth while the female is in labor and there are blood spots on the bedding in the cage (our lab's observation), and persisting close to maximum 6 hours after birth. Therefore, GABA actions at E18.75 might either be depolarizing or hyperpolarizing depending on how far or close to the onset of labor the C-section was performed. It would have been interesting to test the polarity of $GABA_A$ receptors at the time of delivery and at the time when pups should have been born to see if the transient shift in GABA polarity is delayed in mice born by C-section delivery. This could help clarifying if this shift is determined by delivery-induced changes (as suggested by the start of the development of the barrel cortex[486]), or if it is an intrinsic-determined pattern that happens at E19 and is not dependent on birth. If dependent of birth, it would further support that birth is a critical period required for appropriate neuronal development.

In addition, we must also integrate in our understanding the roles of other hormones involved in parturition even though their effect on neuronal development has not been assessed. In fact, progesterone, estrogen, cortisol and catecholamines are present at birth and their interplay could be required for proper development. For example, a balance between neuronal protection (by oxytocin) and stress hormones (catecholamines, AVP) might be required to allow proper adaptation to extrauterine life. Clinical studies have reported that infants born by C-section delivery have lower catecholamines levels than infants born vaginally[183]. We could hypothesize that, since stress molecules are lower, neuronal protection is not required in birth by C-section delivery. Overall, dosing of hormones related to parturition in P0 pups could allow us to assess

hormonal alterations when born by C-section delivery, and test if these deviances are associated with early neuronal brain changes.

Limitations of the use of a mouse model and its correspondence to humans.

As explained previously, several differences exist between humans and animal models. Still, rodent models are essential to understand the fundamental mechanisms underlying the onset of neurodevelopmental disorders, even if their use comes with intrinsic limitations that are important to address. In these species, as gestational length differs (on average 280 days for humans versus 19-21 days for mice), so does development. The Carnegie and Theiler stages comparison suggests that a mouse *in utero* is similar to a human fetus during its first and second trimesters[487]. In addition, the third trimester of gestation in humans corresponds to the early postnatal development in rodents, leading to a human baby brain at birth being comparable to a P7 rat brain[488]. Even though development takes places at different ages in humans and rodents, developmental patterns are conserved across species. Indeed, in the visual cortex, the switch in sensory processing from a "bursting" mode to an adult-like "acuity" mode happens at birth in humans and before eye opening (P14-15) in rats[489]. In the hippocampus, developmental patterns and spontaneous network-driven endogenous activities are also similar despite occurring mostly *in utero* in primates[490,491] and humans[492], but shifting to *ex-utero* in rodents[493]. Furthermore, as previously described, the threshold for pain at birth is higher for vaginal born babies (compared to C-section infants)[120] and for rodents after oxytocin or bumetanide treatments[125], suggesting that the modulation of analgesia at birth by oxytocin and GABA might be preserved across species. These commonalities in the underlying mechanisms between species allowed us to test the hypothesis that birth is a critical period, and that changes in the mode or time of delivery could impact this important developmental milestone.

In our study, we found that C-section delivery induces neonatal alterations (that are aggravated with prematurity) but does not lead to long-term consequences. However, we do not know if these alterations are only due to prematurity, or if they require the combination of both prematurity and C-section delivery. Indeed, epidemiological studies have also reported that prematurity is associated with an increased risk of developing ASD. Our results on eye opening, USVs, and pyramidal neurons morphology around birth give us an insight on the role of prematurity in neonatal development. To further evaluate the role of premature birth without a C-section delivery, we performed preliminary experiments on which we injected mice with mifepristone, a progesterone antagonist, to induce vaginal birth at E17.75 instead of E19. We

were able to evaluate alterations in these mice since no differences were found between vaginal and vehicle-injected mice (Figure 19A-D; Chiesa et al., unpublished data).

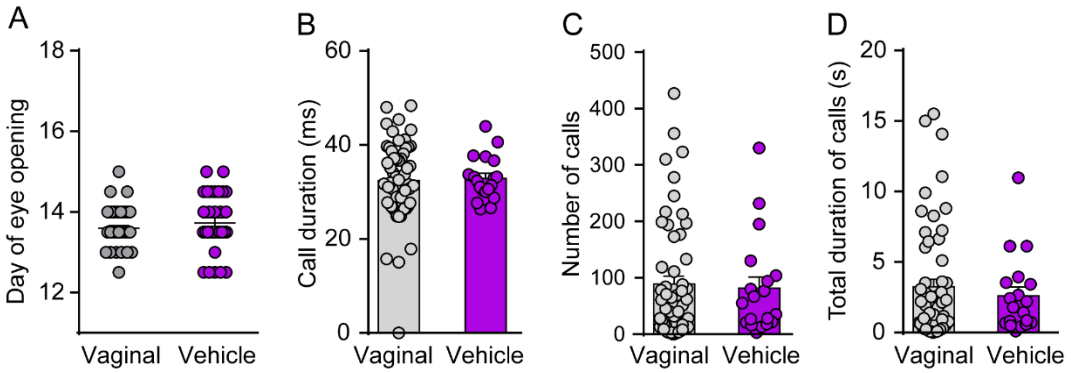

Figure 19. Neonatal development in vaginal and vehicle-treated mice. (A) Postnatal day of eye opening. USVs in mice at P9: (B) Mean duration of calls (ms), (C) Number of calls, and (D) Total duration of calls. (A) n=50 for vaginal, n=31 for vehicle. (B-D) n=55 for vaginal (grey), n=19 for vehicle (purple). Data are presented as mean ± SEM. Data were analyzed with the Mann-Whitney test and no significant differences were observed.

Our results suggest that a delay in eye opening is dependent on prematurity *per se* since mice delivered preterm, either vaginally or by C-section, open their eyes later than vaginal and C-section delivered mice born at term (Figure 20A; Chiesa et al., unpublished data). It is worth noting that, even though not significantly, preterm C-section induces a stronger delay than prematurity by itself. However, prematurity alone did not affect USVs, hence prematurity combined with C-section delivery plays an aggravating role and lead to enhanced alterations (Figure 20B-D; Chiesa et al., unpublished data). Therefore, prematurity and prematurity associated with C-section delivery induce specific developmental alterations. It would be interesting to test if prematurity alone affects CA3 pyramidal neurons morphology, and if alterations are worse in mice born preterm by C-section delivery compared to vaginally.

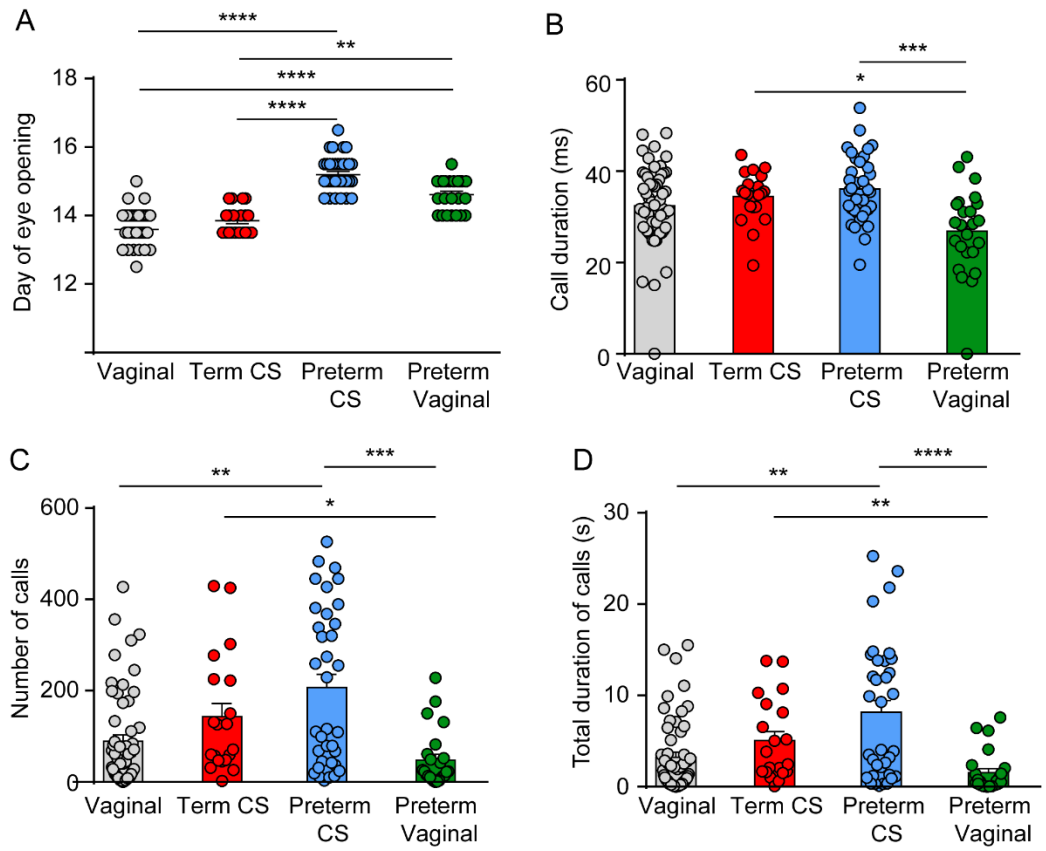

Figure 20. Neonatal development in vaginal, term C-section, preterm C-section, and preterm vaginal born mice. (A) Postnatal day of eye opening. USVs in mice at P9: (B) Mean duration of calls (ms), (C) Number of calls, and (D) Total duration of calls. (A) n=50 for vaginal, n=20 for term CS, n=35 for preterm CS, and n=23 for preterm vaginal. (B-D) n=55 for vaginal (grey), n=20 for term CS (red), n=35 for preterm CS (blue), and n=24 for preterm vaginal (green). Data are presented as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data are analyzed by the Kruskal-Wallis with Dunn's post hoc test.

Relevance of our observations to human epidemiological studies.

Epidemiological studies aimed at evaluating if C-section delivery might be associated with an increased incidence of disorders such as ASD have not yet been conclusive. They remain limited, since only a few of them differentiate the reasons for C-section delivery (such as due to an emergency or planned, or other parameters that took place during the perinatal period). In addition, only one study compared C-section delivery across gestational ages with the probability of developing ASD[252]. In this paper, the authors concluded that the odds for developing ASD were similar for children born by C-section delivery between 36 to 42 weeks of gestation, but were highly variable before 36 weeks of gestation, rendering the conclusion at these ages more difficult. Furthermore, in these studies, children born by C-section delivery were not assessed for their genetic background nor the environment their mother was exposed to during pregnancy, even though ASD has been associated with both factors. For these reasons, as of now, it is impossible to know if the increased incidence of ASD in children born by C-

section delivery is due to one or more external factor(s) or to the surgical act by itself. Our study sheds light on this issue by showing that C-section delivery at term or preterm induces transient developmental delays but does not lead to long-term consequences nor to autistic-like features *per se*. Interestingly, we also show that prematurity could be an aggravating factor, and an earlier prematurity might have led to permanent alterations in our experimental conditions. In humans, prematurity with C-section delivery has already been associated to a higher incidence of respiratory distress syndrome and mortality[194]. Unfortunately, we could not test this possibility in mice as we failed to obtain viable pups born earlier than E17.75. This might be explained by the lung immaturity at earlier gestational ages in mice. In fact, before E17.4, the lymphatic network in lung tissue is not well developed and the surfactant synthesis and secretion, which allow gas exchange, is immature[494]. Finally, we cannot exclude the possibility that a double hit combining C-section delivery with an early or postnatal insult might lead to the increased incidence of ASD observed in some epidemiological studies[250–252,448].

Overall, these transient developmental alterations seen in mice born by C-section delivery might be aggravated by an early in utero, neonatal or postnatal insult. In this scheme, future studies should focus on the hypothesis that delivery is a critical period whose alteration may play an aggravating role to a secondary insult.

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