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Amandine FERNANDEZ

Altérations neurodéveloppementales dans un modèle murin d'Activation Immunitaire Maternelle

Neurodevelopmental alterations in a mouse model of Maternal Immune Activation

Soutenue le 26 Juin 2018 devant le Jury :

Dr. Sonia GAREL	Institut de Biologie de l'Ecole Normale Supérieure	Rapportrice
Dr. Michel VIGNES	Institut des Biomolécules Max Mousseron	Rapporteur
Dr. Nicholas C. SPITZER	UC San Diego	Examinateur
Dr. Fabrice CHRETIEN	Institut Pasteur	Examinateur
Dr. Yehezkel BEN-ARI	Inmed and Neurochlore	Directeur de thèse
Dr. Nail BURNASHEV	INMED	Directeur de thèse

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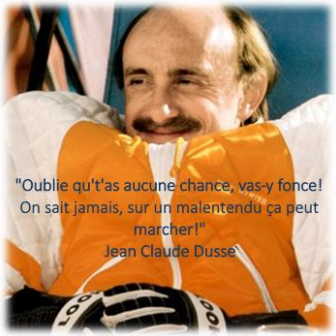

Résumé

Les infections maternelles ainsi que les troubles inflammatoires maternels au cours de la grossesse ont été corrélés à un risque accru de pathologies neurodéveloppementales telles que l'autisme et la schizophrénie chez l'enfant. Les effets de l'exposition in-utéro à une activation immunitaire maternelle (MIA) ont été étudiés dans plusieurs modèles animaux. L'injection de cytokines, mimes viraux ou composants bactériens immunogènes chez la rongeuse gestante, provoque le développement de comportements de type autiste chez les descendants. Cependant, la présence de signatures physiopathologiques dès la naissance est encore méconnue. Le travail présenté ici visait à étudier la présence de séquelles physiologiques chez les souris nées de mères soumises à une MIA in-utéro (induite par le mime viral Poly(I:C), constitué par un ARN double-brin). Nos résultats montrent que la MIA par Poly(I:C) supprime à la naissance le shift dépolarisant vers hyperpolarisant d'action du GABA induit par l'ocytocine dans les neurones pyramidaux hippocampiques de la région CA3. L'application de bumétanide, inhibiteur spécifique de NKCC1, restore ce shift. Les neurones pyramidaux CA3 des nouveaux nés MIA présentent par ailleurs une altération morphologique de leur arborisation apicale, ainsi qu'une plus grande longueur dendritique apicale. De plus, la fréquence des courants post-synaptiques GABAergiques est augmentée chez les descendants MIA à la naissance, ainsi que la synchronicité de décharge entre paires de neurones mesurée en imagerie calcique. Ces altérations physiologiques induites par le MIA à la naissance persistent chez les souris P14-15, puisque l'action du GABA dans les neurones pyramidaux y reste excitatrice. L'activité de réseau reste également augmentée, manifestée par une fréquence accrue des courants spontanés post-synaptiques glutamatergiques. Ces résultats sont en cohérence avec nos travaux réalisés dans deux modèles d'autisme, suggérant que la MIA agit précocement et peut induire une pathogenèse de type autiste. Ainsi, l'exposition à la MIA in-utéro provoque une série d'événements délétères dès la naissance, et persistant durablement.

Abstract

Maternal infections and inflammatory disorders during pregnancy have been correlated with increased risks for developmental brain disorders such as autism spectrum disorders (ASD) and schizophrenia in children. To investigate the effects of in-utero exposure of the foetus to Maternal Immune Activation (MIA), various animal models have been designed. Injections in pregnant rodents of cytokines, viral mimics or bacterial immune components generate offspring with a variety of autistic-like behavioural impairments, but whether patho-physiological signatures are already present in offspring at birth is not known. The work presented here aimed at investigating the early physiological sequels present in mice offspring that were exposed to MIA in utero (induced by the viral mimic double stranded RNA Poly(I:C)). Our results show that Poly(I:C)-induced MIA abolishes the oxytocin-mediated depolarising to hyperpolarising GABA shift in hippocampal CA3 pyramidal neurons at birth, and this was restored by the specific NKCC1 chloride importer antagonist bumetanide. Pyramidal neurons of neonatal MIA pups also present abnormal apical arbour organisation and increased apical dendritic length. In addition, the frequency of spontaneous GABAergic postsynaptic currents is increased in MIA offspring, as well as the pairwise correlation of onset firing of neurons measured with calcium imaging. These physiological alterations of MIA on the day of delivery persist since at P14-15 GABA action remains excitatory and network activity remains elevated with a higher frequency of spontaneous glutamatergic postsynaptic currents. These results are in accordance with our work on two rodent models of ASD, suggesting that MIA acts early and could lead to ASD-like pathogenesis. Therefore, in utero MIA activates in offspring a series of deleterious events manifested already on the day of birth with durable deleterious sequels.

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List of abbreviations

A

ASD: autism spectrum disorder

ATP: adenosine triphosphate

AEP: entopeduncular area

AAC: axo-axonic cells

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate

B

BDNF: brain-derived neurotrophic factor

BBB: blood-brain-barrier

BC: basket cells

BiC: bistratified cells

C

$[Cl^-]_i$: intracellular concentration of chloride ions

CA: cornu-ammonis

CRH: corticotropin-releasing hormone

CNS: central nervous system

CCCs: cation-chloride co-transporters

CP: cortical plate

CGE: caudal ganglionic eminence

CX3CR1: CX3C chemokine receptor 1

CPe: choroid plexus epithelium

CR: calretinin

CCK: cholecystokinin

CB: calbindin

D

DA: dopamine

DF_{GABA}: $GABA_A$ receptor driving force

DG: dentate gyrus

Dlx1/2: distal-Less Homeobox1/2

E

E: embryonic day

EC: entorhinal cortex

EEG: electroencephalography

eGFP: enhanced green fluorescent protein

eIPSCs/eEPSCs: evoked inhibitory/excitatory post-synaptic currents

F

FRX: fragile X

G

GABA: γ-aminobutyric acid

$GABA_AR$: $GABA_A$ receptors

GAD67: glutamic acid decarboxylase 67

GDP: giant depolarising potential

GFAP: glial fibrillary acidic protein

GluR: glutamate-receptor

H

HPA: hypothalamic-pituitary-adrenal axis

I

I.P.: intra-peritoneal

I.V.: intra-venous

IFNβ: interferon β

IL-x: interleukin-x (1α; 1β ; 4; 6; 8 ; 9; 10; 17)

IZ: intermediate zone

K

KCC2: K^+-Cl^- cotransporter 2

L

Lhx6: LIM/homeobox 6

LI: latent inhibition

LPS: lipopolysaccharide

LTD: long-term depression

LTP: long-term potentiation

LGE: lateral ganglionic eminence

M

MBP: myelin basic protein

mGluR: metabotropic glutamate receptor

MIA: maternal immune activation

mIPSCs/mEPSCs: miniature inhibitory/excitatory post-synaptic currents

mPFC: medial prefrontal cortex

mRNA: messenger ribonucleic acid

MZ: marginal zone

MGE: medial ganglionic eminence

N

NAC: nucleus accumbens

NF-κB: nuclear factor-kappa B

NGF: nerve growth factor

Ngn2: neurogenin2

NKCC1: Na^+-K^+-Cl^- cotransporter 1

NMDA: N-methyl-D-aspartate

NPY: neuropeptide-Y

NSC: neural stem cell

O

O-LMC: oriens lacunosum moleculare cells

OT: oxytocin

P

P: postnatal day

PFC: prefrontal cortex

PLP: proteolipid protein

POA: preoptic area

Poly(I:C): polyinosinic-polycytidylic acid

PP: preplate

PPI: prepulse inhibition

PU.1: PU box binding transcription factor 1

PV: parvalbumin

R

RLN: reelin

S

S.C.: subcutaneous

SB: subiculum

SFB: segmented filamentous bacteria

sIPSCs/sEPSCs: spontaneous inhibitory/excitatory post-synaptic currents

SOM: somatostatin

SPAs: synchronous plateaus assemblies

sPSC: spontaneous post-synaptic currents

SVZ: subventricular zone

T

TH: tyrosine hydroxylase

TLR: toll-like receptor

TNFα: tumour necrosis factor α

TrkB: tropomyosin receptor kinase B

V

VGE: ventral ganglionic eminence

VIP: vasoactive intestinal peptide

VPA: valproic acid

VZ: ventricular zone

Introduction

Nature builds.

One common feature shared by almost all materials, organisms, objects, is the necessity of a building procedure allowing for the acquisition of stability. In biology, this progressive building forms the complex process of development.

The biological development is a gradual complexification pipeline which can grossly be defined by three main steps: cell generation, cell differentiation and hierarchical organisation, activity onset and tuning.

Developmental phases are associated with incomparably high plasticity balanced by a symphony of regulating factors. As a result, the reverse face of the coin is an increased vulnerability to perturbation along this program, potentially leading to irreversible alterations. Neurodevelopmental disorders reflect the crucial importance of these processes. Perturbed development for an organism, will ultimately lead to atypical activity. However, one can hypothesise that the early identification of an imbalance and attempting its restoration before the closure of critical periods might reduce the deleterious consequences ultimately leading to pathology. On this line, previous work carried out in our lab in two animal models of autism showed the lasting benefits brought by perinatal restoration of imbalanced neuronal chloride homeostasis.

Accordingly, this thesis aimed at investigating the effects induced by a perturbation of early brain development by Maternal Immune Activation, a known model of Autism Spectrum Disorders (ASD), during the critical period of birth.

To dwell into my thesis work and knowing that we will investigate an early developmental insult, this introduction will start with a brief description of brain development.

Background

I – Central nervous system development

Although brain plasticity occurs throughout the lifespan[1], the early development of the nervous system occurs during the foetal period and first years of life in Humans. During this extensive period, the primordial nervous system emerges from undifferentiated precursor cells that will rapidly divide and differentiate, and thus provide the substrate for subsequent formation of axonal pathways. To better understand the consequences of MIA, a risk factor for neurodevelopmental disorders, the following section will give a description of brain and hippocampus pre-natal development.

1) General embryology

Following fertilisation, rapid cell divisions in the mammalian embryo subsequently form an embryonic disc composed of three layers of cells: (1) the endoderm (inner layer) gives rise to internal organs forming digestive and respiratory systems, (2) the mesoderm (middle layer) forms muscles and skeletal systems, and (3) the ectoderm (outer layer) from which originates the CNS (Figure 1). During gastrulation, mesoderm and endoderm invagination define the midline and anterior-posterior, dorsal-ventral axes of the embryo. A distinct cylinder of invaginated mesodermal cells then extends inward from mid-anterior to posterior of the embryo along the midline, forming the notochord. Under the inductive signals from the notochord, the neuroectoderm located directly above the notochord then thickens into a distinct columnar epithelium: the neural plate[2].

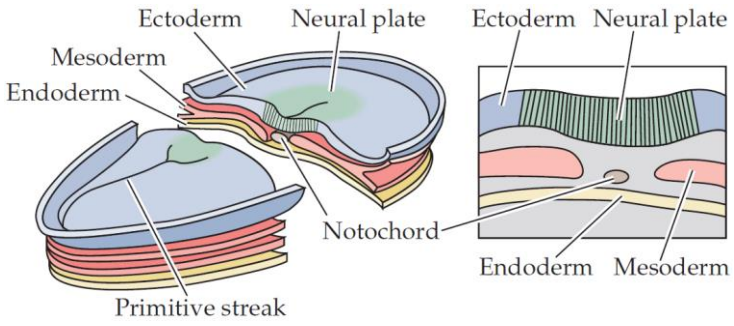


Figure 1 – Late gastrulation and early neurulation of the mammalian embryo. Adapted from Purves D. *et al.* (2004)[2]

During neurulation, the neural plate progressively folds upon itself and closes to form the neural tube. In rodents, closure of the neural tube starts around E8.5 and is completed by E9 except for the roof of the myelencephalon[3].

The head of the neural tube then differentiates into three vesicles: the forebrain (i.e. prosencephalon), midbrain (i.e. mesencephalon), and hindbrain (i.e. rhombencephalon). Subsequently, the prosencephalon further partitions into a rostral telencephalon and dorsal diencephalon. The rhombencephalon divides into rostral metencephalon and caudal myelencephalon. From these primitive territories then emerge the rudiments of brain sub-structures (Table 1)[2,4].

The neural plate and the neural tube are formed by a single layer of apical-basal polarised cells, the neuroepithelial cells. During their cell-cycle, the nuclei of neuroepithelial cells migrate up and down along their apical-basal axis, giving to the neuroepithelium a layered 'pseudostratified' appearance[5].

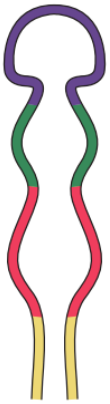
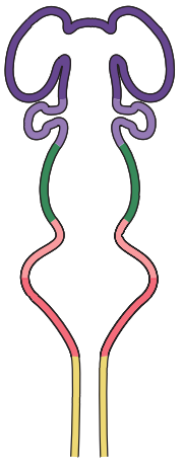
Three-Vesicle Stage (Primary Brain Vesicles)		Five-Vesicle Stage (Secondary Brain Vesicles)		Mature Structure
	Forebrain - Prosencephalon		Telencephalon	• Cerebral cortex • Basal ganglia • Hippocampus • Amygdala • Olfactory bulbs
			Diencephalon	• Thalamus • Hypothalamus • Optical tracts • Retina
	Midbrain - Mesencephalon		Mesencephalon	• Midbrain • Tegmentum
	Hindbrain - Rhombencephalon		Metencephalon	• Pons • Cerebellum
			Myelencephalon	• Medulla oblongata
	Caudal part of the neural tube		Caudal part of the neural tube	• Spinal cord

Table 1 – Neurulation. Adapted from Purves D. *et al* (2004), and Li G.(2013)[2,4]

2) Neurogenesis

Overview

In mice, a progressive transition from neuroepithelial cells to more fate-restricted neural progenitors, namely radial glial cells, occurs between E10 and E12[5].

Neurogenesis from radial glial cells takes place in two major proliferative zones located in the telencephalon. (1) The ventricular zone of the dorsal telencephalon (VZ), that surrounds the lumen of the neural tube and will later become the brain ventricles, gives rise to major neuronal populations of the neocortex comprising the excitatory pyramidal neurons[6]. (2) The medial and caudal ganglionic eminences (MGE, CGE), transitory developmental structures located in the ventral telencephalon, which provide neuronal cells of deep brain nuclei and cortical interneurons, and the preoptic area (POA) which provides a minority (10%) of the neocortical interneurons[7].

First, the precursors of the VZ divide to form either new stem cells or postmitotic neuroblasts that will further differentiate into neurons. Second, as they become postmitotic, neuroblasts migrate out of the VZ towards their final position in the cortical plate to undergo terminal differentiation. Neuroblasts can migrate either following a tangential migration under the guidance of adhesion molecules, or a radial migration by crawling along the scaffold provided by radial glial cells[6].

During later stages of neurogenesis, a subventricular zone (SVZ) emerges above the VZ. By opposition to the VZ, progenitor cells of the SVZ do not migrate, and remain in the SVZ as they divide. After the completion of neuronal migration, the VZ reduces progressively, and disappears. However, a pool of stem cells will remain in the SVZ and keep its proliferation capacity all through life (for a review, see ref.[6]).

Ganglionic eminences start to form by protruding into the lateral and third ventricle around E11 in mice, giving rise to the MGE and LGE by E12. The CGE is formed by the posterior area where the LGE and MGE fuse together[8]. These domains express specific genes that define their identities and allow the specification of interneurons and oligodendrocytes, such that progenitor cells form distinct pools that are spatially segregated. Within the ganglionic eminences, neural progenitors are also organised into VZ and SVZ[7]. In the VZ, many progenitor cells have the typical features of radial glial cells, whereas in the SVZ can be found intermediate

progenitor cells likely arising from asymmetric division of radial glia[9]. After generation, interneurons then migrate tangentially to reach their final position within the neocortex. By E14-15, the sulcus between the eminences begins to disappear, to fade into the wall of the lateral ventricle by the end of the embryonic development[8].

Migration

The first neurons to be generated leave the proliferative zone and form the preplate (PP). The wave of neurons migrating afterwards splits the PP region into two transient brain layers: the marginal zone (MZ) directly beneath the pial surface, and the subplate above the VZ. Between the MZ and the subplate, neurons that are generated accumulate within the PP and form the cortical plate (CP)[10]. Consequently, consecutive waves of migrating neurons bypass the previous waves of neurons to reach the most superficial position within the developing cortex (Figure 2), such that earlier migrating neurons constitute deepest cortical layers, and later migrating neurons form successively more superficial layers; in an "inside-out" fashion. In the MZ, Cajal-Retzius neurons born in the first wave of migration produce reelin, a molecular signal involved in the signalling pathway responsible for the end of migration: once migrating neurons reach the zone containing the reelin signalling, they receive the cue to stop migrating. The superficial MZ therefore constitutes the future cortical layer I[10].

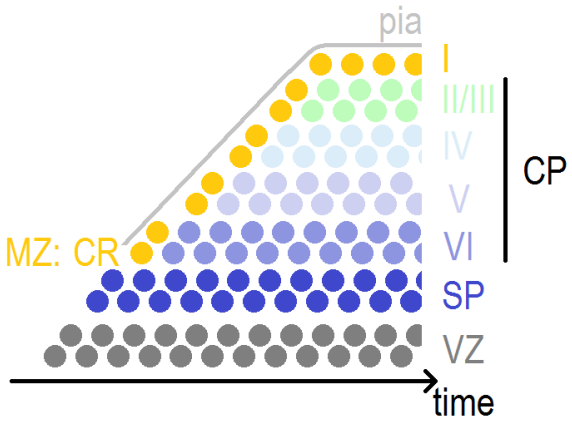


Figure 2 – During development, waves of neuronal migration progressively build cortical layering in an "inside-out" spatiotemporal gradient. MZ = Marginal Zone; CR = Cajal-Retzius cells ; SP = Subplate; VZ = Ventricular zone; CP = cortical plate ; II-VI = developing cortical layers. Adapted from Stiles, J. & Jernigan, T. L. (2010)[10].

3) Brain cell populations: Neurons

Neurons are excitable and secretory cells of the CNS. They form complex networks by establishing synaptic contacts, that allow the transmission and processing of information[11]. In

the CNS, various types of neurons can be defined according to the type of neurotransmitter released at their synapse (Table 2). The specificity for neurotransmitter expression is defined during development by intrinsic transcription factors[12,13], extrinsic signalling molecules[14,15], or modulation of the neuron's spontaneously generated patterns of Ca^{2+} spikes. This latter mechanism allows neurons to 'switch' the expressed neurotransmitter without changing their intrinsic identity[16,17].

In the adult, the diversity of neurotransmitters and associated receptors allow for the modulation of neuronal activity, and the generation of a finely tuned network output.

However, with regards to the work carried out in this thesis, the following section will focus on the neocortical neurons of the CNS described here after.

NEUROTRANSMITTER/NEUROMODULATOR	MOST COMMON POSTSYNAPTIC EFFECT
Acetylcholine	Excitatory
Glutamate	Excitatory
GABA	Inhibitory
Glycine	Inhibitory
Catecholamines • Epinephrine • Norepinephrine • Dopamine	Excitatory
Serotonin	Excitatory
Histamine	Excitatory
ATP	Excitatory
Neuropeptides	Excitatory and inhibitory
Endocannabinoids	Inhibits inhibition
Nitric oxide	Excitatory and inhibitory

Table 2 – Major neurotransmitters and neuromodulators and their most common postsynaptic effects in the adult. Adapted from Purves D. *et al* (2004)[2].

The vast majority of cortical neurons, referred to as 'principal' neurons, accounts for pyramidal excitatory glutamatergic neurons (70 to 80%) projecting to distant brain areas[18]. Although some variability exists regarding their soma shape, projection site and dendritic arbour morphology, pyramidal cells show relatively homogeneous anatomical, physiological, and molecular properties. Typically, pyramidal cells soma shape is pyramidal or ovoid, with an arborized prominent apical dendrite directed radially towards the pia matter at the upper pole. From the base emerge basal dendrites directed radially or downward[18] (Figure 3). Cortical projection neurons originate from the dorsal telencephalon and migrate radially into the

cortex. Laminar distribution of these neurons within the mature neocortex defines the area targeted by their axonal projections[19].

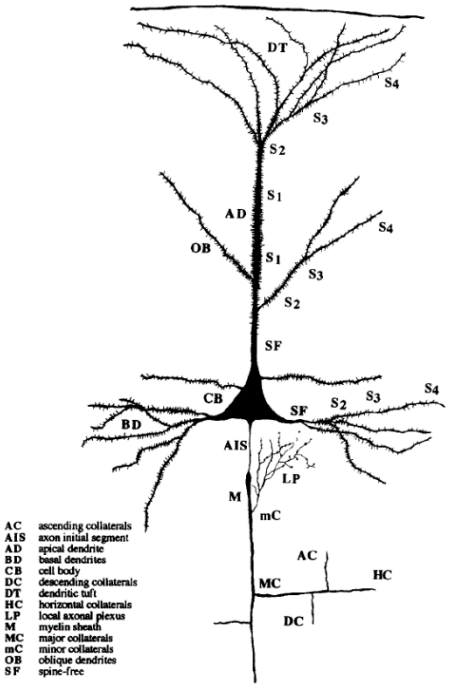


Figure 3 – Schematic diagram of a typical pyramidal cell. From DeFelipe and Fariñas (1992)[18].

Interneurons constitute the remaining 20 to 30% of cortical neurons. By contrary to pyramidal neurons, interneurons are mainly inhibitory in the adult CNS and use γ-amino-butyric acid (GABA) and Glycine as their transmitter. In addition, interneurons' dendritic arbours and axon project only within the cortex; making them 'local circuit neurons'[20].

Morphologically, mature inhibitory interneurons have aspiny dendrites, and receive both excitatory and inhibitory synapses onto their somata. In addition, interneurons form an extensively heterogeneous population of cells expressing diverse molecular, physiological, synaptic and morphological characteristics. Consequently, differential axonal arborisation morphology and preferential establishment of synaptic contacts onto either axon, soma, or dendritic arbour has been used as a criteria for classification (Figure 4)[20]. Functionally, this targeting specificity allows for the precise spatiotemporal control of principal neurons, notably regarding the timing of firing and synchronisation of network activity[21]. Coherently, another classification of interneurons subtypes relies on their intrinsic firing properties, reflective of their activity and potential role within cortical circuits[22].

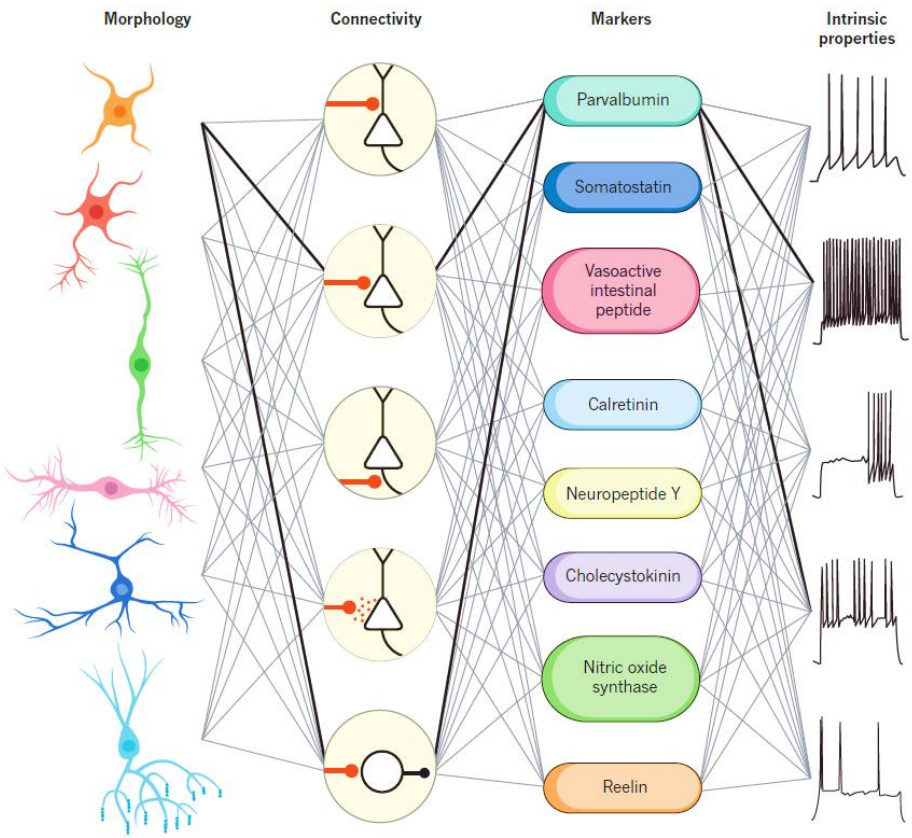


Figure 4 – Interneuron diversity. Interneurons subtypes show morphological differences, express diverse molecular markers, have preferential localisation for synaptic contact, and show different firing patterns. Adapted from Kepecs, A. & Fishell, G. (2014)[23].

The majority of telencephalic GABAergic interneurons migrate tangentially from the medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE). Lateral ganglionic eminence (LGE) and preoptic area; also designated under the term anterior entopeduncular area (POA; AEP, respectively) contribute to a lesser extent, mainly regarding specialised subpopulations of interneurons[22,23] (Table 3). Furthermore, during telencephalic development, tangentially migrating interneurons were shown to follow distinct spatial and temporal routes. Importantly, in rodents, interneurons expressing parvalbumin or somatostatin together account for around 70% of cortical interneurons and derive primarily from the MGE[24]. Bipolar cells expressing calretinin represent nearly 15% of the total interneuron population, and seemingly emerge exclusively from the dorsal CGE[24]. However, these markers appear later in the development. This will be further described in section III.3.

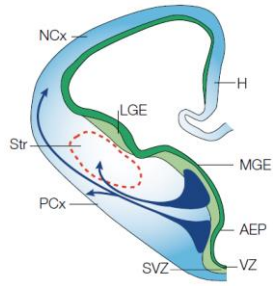
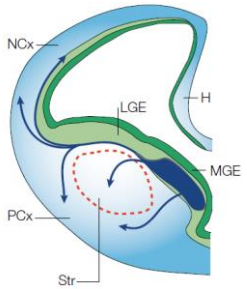
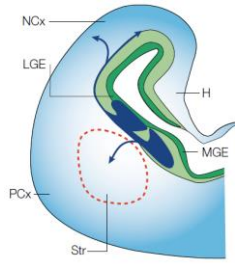
SCHEMATISED MOUSE EMBRYONIC TELENCEPHALON	EMBRYONIC AGE (MOUSE)	ORIGIN	ROUTE	DESTINATION
	E11.5	MGE POA/AEP	Superficial	• Developing striatum • Cortical marginal zone • Cortical subplate
	E12.5-E14.5	MGE	Deep or superficial	• Developing striatum • Subventricular-zone, Lower intermediate zone • Subplate, then extend into the cortical plate
		LGE	Rostral migratory stream	Olfactory bulb
	E14.5-E16.5	LGE MGE	Deep	Cortex

Table 3 - Three spatially and temporally distinct tangential migratory routes of immature interneurons can be distinguished. Schemas represent transverse sections through the mouse embryonic telencephalon. Around E11.5, interneurons arise primarily from the medial ganglionic eminence (MGE) and the anterior entopeduncular area (AEP), and follow a superficial route to the cortex. E12.5-E14.5 is the peak of migration: interneurons migrating to the cortex arise primarily from the MGE and follow a deep route to the developing striatum (Str). However, some interneurons also migrate superficially. At E14.5-E16.5, cells migrating to the cortex might also arise from the lateral ganglionic eminence (LGE) and follow a deep route. H, hippocampus; NCx, neocortex; PCx, piriform cortex; VZ, ventricular zone; SVZ, subventricular zone. Adapted from Marín, O. & Rubenstein, J. L. R. (2001)[25].

4) Brain cell populations: Glia

Glial cells were first described by Rudolf Virchow as a "connective tissue binding nervous elements together" (1858), before their cellular nature was established by Camillo Golgi[26]. Glial cells of the brain encompass three types of cells: astrocytes and oligodendrocytes generated from neural precursor cells, and microglia arising from a non-nervous peripheral source. Altogether, this "glial network" maintains an optimal environment for neuronal function.

Interactions between neurons and glia during development shed light on the supporting role of glia encompassing axonal growth, synaptogenesis, synaptic pruning and other processes[272628].

Astrocytes & oligodendrocytes

Astrocytes are the most abundant cell type in most parts of the brain[29]. Protoplasmic astrocytes can be found uniformly distributed in the grey matter, whereas fibrous ones localise within the white matter along the nervous fibres bundles[27]. They are multitasked supporting cells of the CNS, involved in blood flow control, energy metabolism, potassium buffering, neurotransmitter clearance from the synaptic cleft and recycling, control of synapse formation, function and elimination[30]. They also provide glutamatergic and GABAergic neurons with glutamine, and are able to release neuroactive factors such as transmitters, neuropeptides, growth factors, and steroids[31]. Although not excitable, astrocytes were reported to show transient elevations of intracellular calcium concentrations ($[Ca^{2+}]_i$) in response to glutamate either within individual cells, or propagating from a cell to its neighbours and rapidly transmitted to surrounding neurons[32]. This suggested that astrocytic calcium signalling would have a functional role in the modulation of neuronal activity.

Importantly, astrocytes can react to all forms of CNS perturbations, from subtle disturbances to pathological injury or disease, by adopting different degrees of reactive astrogliosis: mild to moderate, severe, and glial scar formation[33]. Reactive astrocytes can protect CNS cells and tissue in various ways, including by the uptake of potentially excitotoxic glutamate, the protection from oxidative stress via glutathione production, the facilitation of blood brain barrier (BBB) repair, the reduction of oedemas and the stabilisation of extracellular fluid and ion balance (see[33] for a review).

Oligodendrocytes on the other hand, are the myelinating glial cells of the CNS, and are also capable of releasing neuroactive compounds such as steroids and nerve growth factor (NGF)[31,34].

During development, stem cells of the VZ chronologically allow the generation of neurons (neurogenesis) in a first time, and glial cells (gliogenesis) in a second time. This gliogenesis also follows a developmental chronology: astrocytes are produced first, oligodendrocytes second (Figure 5). The “gliogenic switch” of neural stem cells towards an astrocyte precursors fate depends on the induction of a transcriptional cascade inducing specification[35]. Afterwards,

astrocyte precursors migrate away from the germinal centres and differentiate while starting to express the astroglial marker GFAP. Gliogenesis starts during late embryogenesis and continues in postnatal stages[30,35].

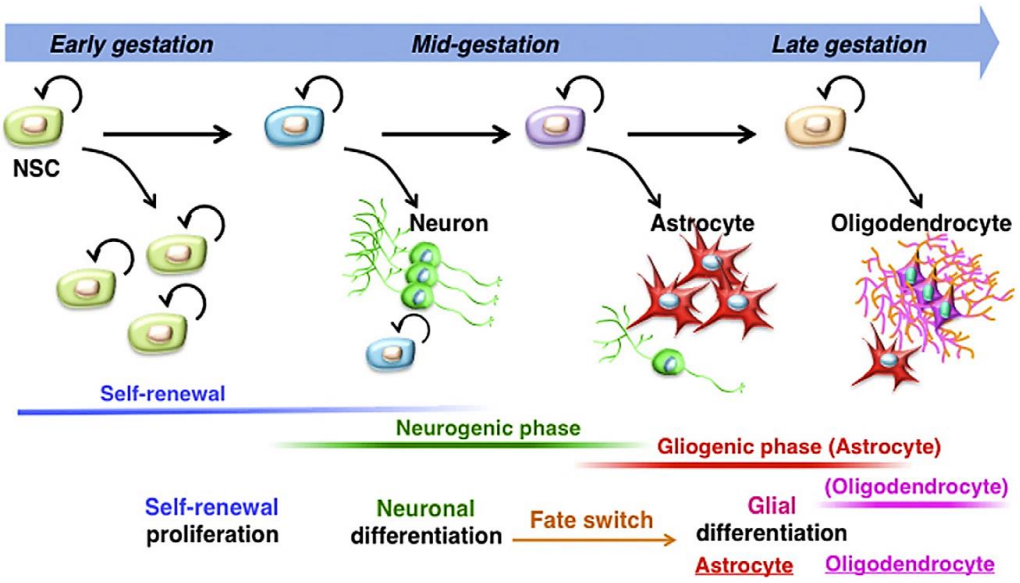


Figure 5 – Schematic diagram depicting developmental stage-dependent neural stem cells (NSCs) differentiation in the mammalian embryonic CNS. At early embryonic stages, NSCs expand their pool by self-renewing symmetric divisions. NSCs first acquire the potential to differentiate into neurons at mid-gestation, then into glial cells from late to perinatal stages. From Murao, N., Noguchi, H. & Nakashima, K. (2016)[36].

Microglia

A microglial cell is both a glial cell of the CNS and a mononuclear phagocyte, which belongs to the haematopoietic system and is involved in inflammatory and immune responses. Microglial cells continuously monitor and maintain brain homeostasis in physiological conditions, by extending motile processes. In this steady state, they contribute to the modification and elimination of synaptic structures[37]. Upon perturbation of brain homeostasis through ischemia, infection, degeneration or injury, microglia secrete chemokines, and acquire an activated state endowing them with phagocytic capacity[38]. All through life in the absence of pathogenic events, the pool of microglial cells acquired before closure of the blood-brain barrier (BBB) self-renews within the brain without external contributions[37]. Indeed, during gestation, primitive myeloid precursor cells arise from the yolk sac at E6.5-E7.5 and migrate to reach the foetal brain around E9 via the blood stream and ventricles. As a consequence, they first cluster around subcortical regions such as the hippocampus and corpus callosum, and migrate from there to populate the entire brain[39]. Time-lapse imaging and

immunohistochemistry of fixed brain slices from transgenic knock-in mice expressing eGFP under the control of the monocyte marker CX3C chemokine receptor 1 (CX3CR1) showed that microglial cells invade the foetal cortex starting from E11.5 to E17.7 and tend to accumulate close to dying cells in the prospective choroid plexus, where they may acquire phagocytic phenotype and clear apoptotic cells debris[40]. The microglial pattern of invasion in the foetal brain follows consecutive slow and fast waves of migration, during which microglial morphology evolves towards a more ramified phenotype (figure 6)[40]. During development, microglial cells were also shown to actively support *Lhx6*-expressing interneuron's migration, and to control the axonal outgrowth of dopaminergic neurons in the mouse forebrain[40]. Furthermore, disruption or perturbation of microglia function such as in *Pu.1* knock-out embryos, in which no generation of myeloid cell takes place, or after LPS-induced MIA were shown to result in altered dopaminergic neurons axonal outgrowth and abnormal distribution of *Lhx6*-expressing interneurons[41].

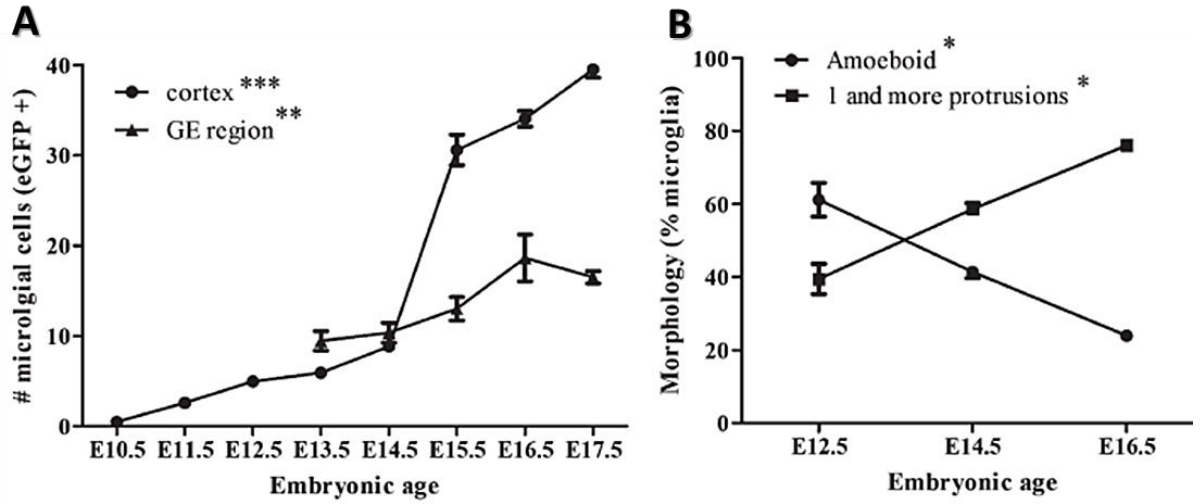


Figure 6 – Quantification of microglial cell invasion into the foetal brain. A. Microglial cells invade the cortex and ganglionic eminences (GE) by successive waves starting from E10.5 in the cortex, and E13.5 in GE. Mild increase of microglia occurs between E10.5 and E14.5, followed by a fast increase after E14.5. **B**. The morphology of microglial cells changes throughout development. At E12.5, microglia have a non-ramified amoeboid form. They progressively develop protrusions/ramifications as the development proceeds. Adapted from Swinnen, N. *et al.* (2013)[40].

5) Brain cell populations: vascular cells

Blood vessels also form a rich network in the CNS. The density of vascularisation varies between grey and white matter, with the cerebral cortex and subcortical grey matter being the

most vascularised compared to white matter. Additionally, capillaries are denser in regions packed with a high number of neurons or presenting a high density of synapses. Brain capillaries form the protective blood-brain-barrier (BBB), allowing the precise control over the substances that leave or enter the brain, except in the circumventricular areas[42]. This precision relies on the expression of a complex network of tight junctions, the expression of specialised transporters on endothelial cells membranes, and a reduced pinocytotic activity.

In brief, the structure of the BBB is composed by a superposition of layers comprising: an endothelial cell making the capillary wall, surrounded by a thin basement membrane (or basal lamina) in which lie pericytes. Perivascular astrocytes then appose their end feet against the continuous basal lamina (figure 7)[43].

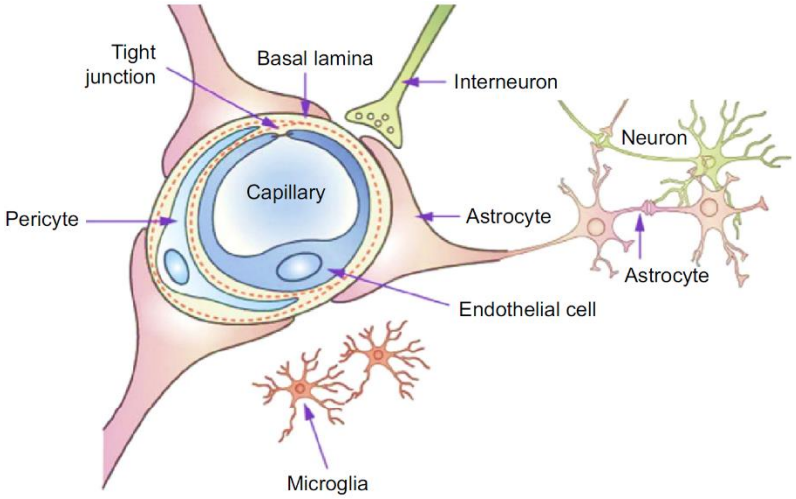


Figure 7 - Schematic of the fully developed blood-brain barrier and associated components of the neurovascular unit. Adapted from Feng, X. *et al.* (2015)[43].

Development

The development of the brain vasculature begins with the entrance of mesodermal angioblasts into the head region, where they cover the entire surface of the neural tube to form the perineural vascular plexus[42]. The vascular system then develops by sprouting from the perineural plexus, radially invades the proliferating neuroectoderm around E11 in rodents, elongates and forms branches that will anastomose to create a plexus of undifferentiated capillaries in the ventricular zone of the developing brain. The vascular sprouts entering the neural tube around E11 are permeable to small hydrophilic substances, but seemingly not to macromolecules[42]. The permeability of the barrier progressively increases as capillaries lose

their fenestrae, inter-endothelial junctions complexify, and angiogenesis increases gradually. Thus in the rat embryo, fenestrations disappear entirely by embryonic day 17[44]. Although BBB permeability to macromolecules at that stage is comparable to that of the adult, small molecules and ions can cross more readily than in the adult brain. However, permeability to ions drops just before birth[42,45].

6) Summary

Brain development comprises a progressive complexification of its structure, and time regulated generation of complementary cell populations. The existence of mixed cell populations at different stages of their maturation allows for cooperative developmental processes, and gradual acquisition of functional brain circuitry (figure 8).

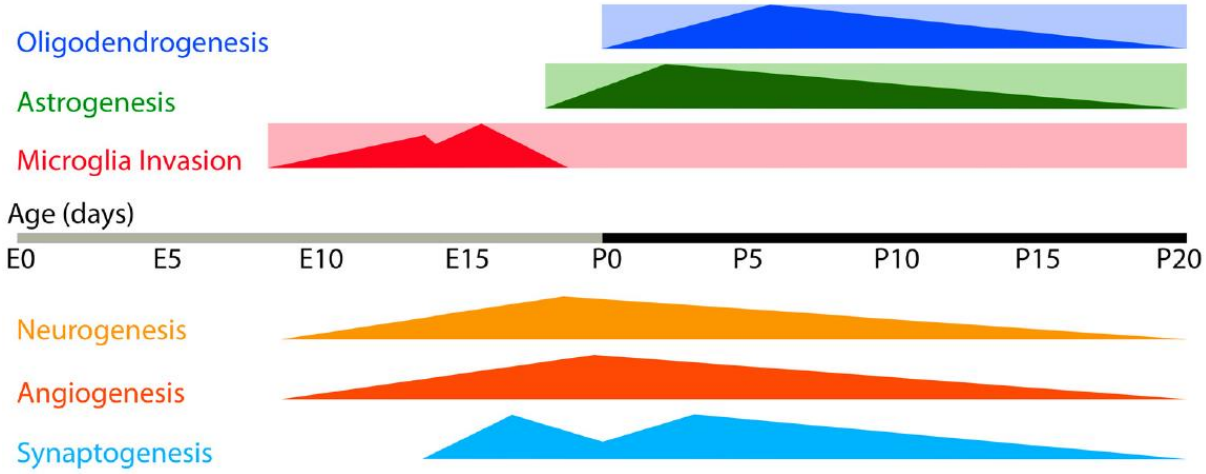


Figure 8 – Timeline of key developmental processes in the embryonic (E) and postnatal (P) mouse brain. Rectangles start at the estimated time when oligodendrocytes, astrocytes, and microglia begin to be present in the brain. Triangles represent the onset and peak of mentioned developmental events. Adapted from Reemst, K. *et al.* (2016)[46].

II – The hippocampus

The hippocampus is a phylogenetically conserved structure of the CNS playing an integral role in learning, emotion, synaptic plasticity, and spatial and declarative memory[45,47,48]. Impairments of this structure are suggested to play a significant role in the pathophysiology of cognitive and psychiatric disorders[49–52].

My thesis studied the consequences of MIA on CA3 hippocampal pyramidal cells and network activity. The following section will give an overview of this structure and embryonic development.

1) Structure

The hippocampus is a major component of the mammalian nervous system located in the medial temporal lobe. Anatomically, the hippocampus is a bilateral, even, long, curved structure formed from the edge of the cerebral cortex that together with the cingulate cortex, olfactory cortex and amygdala, make up the limbic system[53]. The hippocampal region comprises two sets of cortical structures differing by their number of cortical layers, and connectivity schemes: the hippocampal formation and the parahippocampal region.

The hippocampal formation, or hippocampus proper, is a five-layered structure comprising two crescent-like regions: the Ammon's horn, subdivided into CA3, CA2 and CA1 regions (with an additional CA4 region in Human) and the dentate gyrus, itself subdivided into the stratum moleculare and the hilus (Figure 9.A.). Within the hippocampal formation, the principal cell layer is called the stratum pyramidale. The narrow, relatively cell-free layer located deep to the pyramidal cell layer is called stratum oriens. Superficial to the CA3 is the stratum lucidum, occupied by mossy fibers projecting from the dentate granule cells. The stratum radiatum is located superficial to the stratum lucidum in CA3 and the pyramidal cell layer in the CA1 and CA2. The most superficial layer in the hippocampal proper is called the stratum lacunosum-moleculare[11,53].

The parahippocampal region, corresponding to the adjacent cortical areas to which the hippocampus proper is connected, comprises the perirhinal, entorhinal, postrhinal cortices, the presubiculum and parasubiculum (Figure 9.B.)[3].

The following sections will focus on the hippocampus proper.

2) General connectivity

The connectivity between areas of the hippocampal formation has been considered for a long-time as a tri-synaptic loop in which signals propagate first through the dentate gyrus (DG), then to the CA3 layer, then to the CA1 layer, then to the subiculum (SB), and finally exits the hippocampus towards the entorhinal cortex (EC) (DG > CA3 > CA1 > SB >EC) (Figure 9.B.).

However, more recent studies demonstrated the existence of the CA2 input onto CA1 pyramidal neurons independently of CA3. Therefore, transmission of entorhinal input through CA2 would parallel the tri-synaptic loop[54,55]. Additionally, each of the hippocampal regions also contain complex intrinsic circuitry that operate to generate rhythmical activity within each region, as well as extensive longitudinal connections[11,53,56]

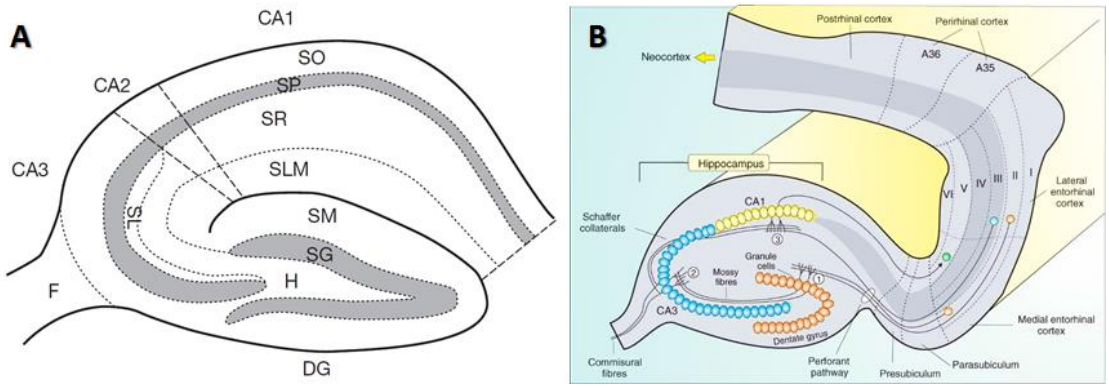


Figure 9 – Schema of hippocampal formation structure and lamination. CA, cornu ammonis; DG, dentate gyrus; F, fimbria; H, hilus; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare; SL, stratum lucidum; SM, stratum moleculare; SG, stratum granulosum. From Li, G. & Pleasure, S. J. (2013)[3]. B. Organisation of the hippocampus and parahippocampal regions, and connectivity. The hippocampal trisynaptic circuit starts with (1) first synapses on the granule cells of the dentate gyrus (orange), that receives input from the perforant fibres. These granule cells send out axons, the 'mossy fibres' that extend into the CA3 region (blue), where they form the (2) second group of synapses by innervating the CA3 pyramidal cells. The axons from the CA3 neurons bifurcate: one part is directed down to the septum (the commissural fibres), the other gives rise to Schaffer collaterals that complete the trisynaptic circuit by innervating the pyramidal neurons (3) in the CA1 region (yellow). From Berridge, M. J. (2014)[57].

3) Development

A summary of the course of the hippocampal histological development from the initial closure of the neural tube is described in the table hereafter (Table 4).

Pyramidal cells

In mice, hippocampal pyramidal cells arise from the radial glial cells of the hippocampal neuroepithelium at E14–E15 for CA3, and at E15–E16 for CA1[58,59]. After leaving the neuroepithelium, young neurons pause for four days in the subventricular (SVZ) and intermediate zone (IZ). In the meantime, hippocampal alveolar channels form and receive hippocampal fibres, and the limbs of the granular layer of the dentate gyrus assemble to form the hilar region in which CA3 pyramidal cells will settle[60]. At the end of their migration, pyramidal cells settle into the hippocampal plate; the presumptive stratum pyramidale, which

splits the preplate into the subplate (the prospective stratum oriens) and molecular zone (MZ). The MZ then further splits into inner and outer MZ, corresponding to the prospective stratum radiatum and stratum lacunosum moleculare, respectively[60]. During their migration, a non-vesicular release of GABA and glutamate[61] influences pyramidal cells motility through the activation of $GABA_A$ and NMDA receptors[62]. As in the cortex, pyramidal neurons migrate through an 'inside-out' fashion: early-born neurons constitute the deep location of the pyramidal layer and late-born neurons settle at more superficial locations. By contrary to the cortex, the successive waves of migration form a single relatively homogeneous layer of pyramidal cells instead of forming distinct layers of neurons[63]. However, the maturation of pyramidal cells is not homogeneous, and follows a stepwise acquisition of synaptic input correlated with the morphological growth of the apical tree[64]. Accordingly, three populations of pyramidal neurons were shown in the rat hippocampal CA1 area at birth (Figure 10. A.): 80% of silent neurons, with no or little apical dendrite; 10% of neurons with spontaneous post-synaptic currents (sPSCs) only mediated by $GABA_A$ receptors ($GABA_AR$) with a small apical dendrite; and 10% of neurons showing both $GABA_AR$ and glutamate-receptor (GluR) mediated sPSCs, with an arborized apical dendrite reaching the stratum lacunosum moleculare. Thus, GABAergic synapses are established prior to glutamatergic ones on pyramidal neurons, the latter being dependent on a development of the apical dendrite sufficient to reach the stratum lacunosum moleculare[64].

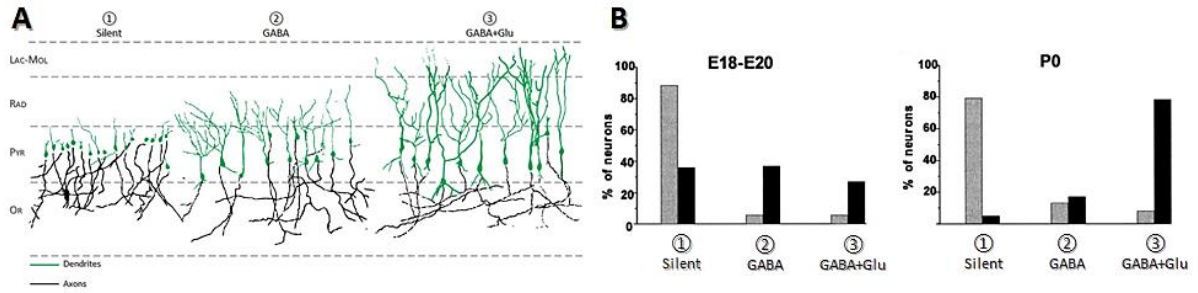


Figure 10 – Hippocampal pyramidal neurons present heterogeneous degrees of maturation around birth. A. Camera lucida reconstruction of biocytin-filled pyramidal neurons in the rat hippocampus at birth. 3 groups of neurons were identified, each group having similar physiological and morphological properties. ① Pyramidal neurons with the less advanced degree of maturation have no or little apical dendrite, do not respond to stimulation and show no sPSC. ② Intermediate neurons have small apical dendrite and only GABA sPSCs. ③ The more mature pyramidal neurons have apical dendrite reaching the lacunosum moleculare layer, and $GABA_A$+Glutamate sPSCs. Adapted from Tyzio, R. *et al.* (1999)[64]. **B.** Proportions of pyramidal neurons (**grey bars**) and interneurons (**black bars**) shortly before birth (E18-E20) and on the day of birth (P0) in the rat hippocampus. At both ages, most pyramidal neurons are silent, whereas most interneurons show active synapses. Adapted from Hennou, S. *et al.* (2002)[65].

Histological development

EMBRYONIC AGE/REF	HIPPOCAMPAL DEVELOPMENT (MOUSE)
E8.5[63]	Beginning of closure of the rostral neural tube from the presumptive forebrain–midbrain junction toward the rostral end.
E9[66]	• Closure of the rostral neural tube is complete, except for the roof of the myelencephalon. • The cortical hem starts to express the morphogenetic factors WNT and BMP.
E10.5[67,68]	• Midline of the telencephalic vesicle invaginates to form the medial walls of the two telencephalic hemispheres. • Appearance of the 'choroid plaque' at the midline choroid plexus epithelium presumptive (CPe). • Neurogenesis begins.
E12.5[66]	Four tissues can be identified in the medial wall of the telencephalon (from dorsal to ventral): • embryonic cerebral cortex • the cortical hem • junctional epithelium • CPe
E14[69]	Hippocampal pyramidal cells arise from the radial glia in the germinal ventricular zone of the hippocampal primordium
E14.5[70]	Hippocampal field patterning emerges in the medial telencephalic wall.
E15[71]	Hippocampal fibres develop.
E16.5[71]	Entorhinal afferents reach CA1.
E17.5[71]	Septum afferents arrive in the mouse hippocampus.
E18[71]	Commissural fibres enter the contralateral hippocampus.
E19.5[71]	Entorhinal cortex afferents reach the dentate gyrus.

Table 4 – Histological development of the mouse hippocampus.

Interneurons

In mice, most interneurons from CA1 and CA3 are generated at E12–E13[72], and the majority of interneurons of the dentate gyrus at E13–E14[73]. Thus, the peak of genesis for interneurons occurs prior to the peak of genesis of pyramidal cells. Hippocampal interneurons mainly arise from the medial (MGE) and caudal ganglionic eminences (CGE), with contribution of the preoptic area (POA). Specifically, MGE-derived interneurons give rise to the majority of parvalbumin (PV) and somatostatin (SOM) expressing interneurons, whereas calretinin (CR) and vasoactive intestinal peptide(VIP)-expressing subtypes mainly come from the CGE[11,59]. Besides, the variety of peptide and calcium-binding proteins expressed by interneurons subtypes follow a postnatal maturational profile. Globally, neuropeptide expressions are first detected between E18, as for neuropeptide-Y (NPY)-expressing, and P5 for VIP-expressing

interneurons in mouse hippocampus and reach adult levels around P20. PV immunoreactivity appears at P4–7, first in CA3, then in CA1[59]. PV mature 'fast-spiking' electrophysiological phenotype relies on voltage-gated potassium channels of the Kv3 family, that start being expressed at the end of the first postnatal week and increase progressively up to P40. Electrical coupling of PV neurons through gap junctions is also developmentally regulated during postnatal development (Figure 11). Namely connexin 36, a major connexin subunit in neurons, increases until P7–P16, and then decreases towards adult levels[59].

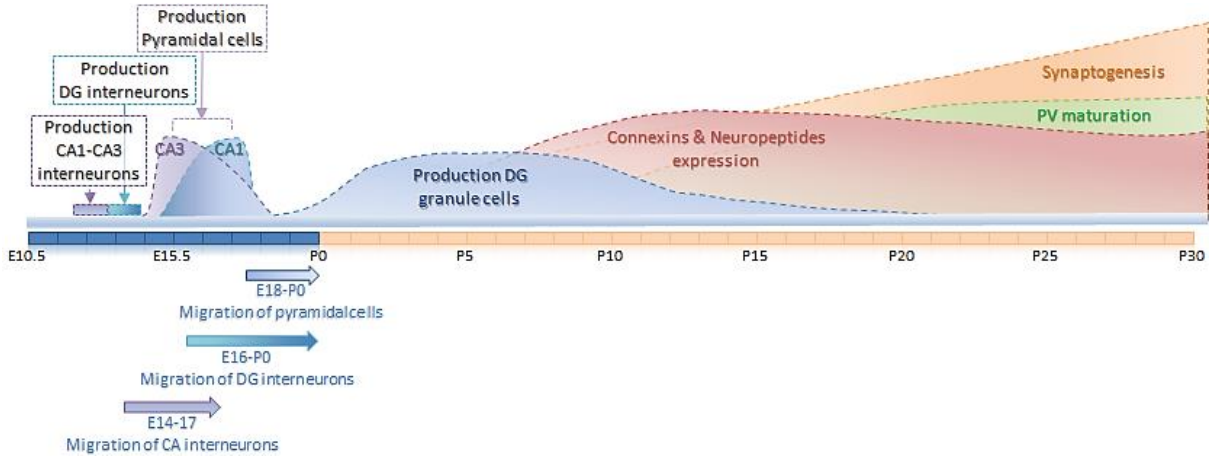


Figure 11 – Generation of hippocampal principal cells and interneurons, and maturation of interneurons molecular markers. Hippocampal interneurons are produced prior to pyramidal cells, with CA1-CA3 interneurons and principal cells also produced before dentate gyrus (DG) granule cells. Accordingly, interneurons migrate first and reach targeted hippocampal regions prior to principal cells. Similarly, CA neurons settle in the hippocampal layer before granule cells reach the DG. Subgroups of hippocampal interneurons are characterised by the expression of specific neuropeptides, that start to be expressed at late embryonic stages in the hippocampus and exhibit a dynamic maturation pattern of expression during the postnatal period. Adapted from Danglot, L. *et al* (2006)[59].

In the adult hippocampus, the four resulting types of interneurons are all GABAergic, present in either layer, and finely tune principal cells' activity by targeting specific subdomains (Figure 12). Basket cells (BC) are either PV or cholecystokinin (CCK)-VIP positive. BC innervate the soma and proximal dendrites located in stratum pyramidale and radiatum. Cell bodies of basket cells are mainly located in stratum pyramidale. Bistratified cells (BiC) are SOM and calbindin (CB) positive and innervate both apical and basal dendrites on their proximal part located in stratum radiatum and oriens. Cell bodies of bistratified cells locate in stratum oriens/radiatum or stratum pyramidale. Oriens lacunosum moleculare cells (O-LMC) are SOM positive, and innervate distal apical dendrites located in stratum lacunosum moleculare. Cell bodies of O-

LMC are located in stratum oriens. Axo-axonic cells (AAC) are also called chandelier cells, express PV and innervate exclusively the axon initial segment. Their cell bodies are located within or near stratum pyramidale[11].

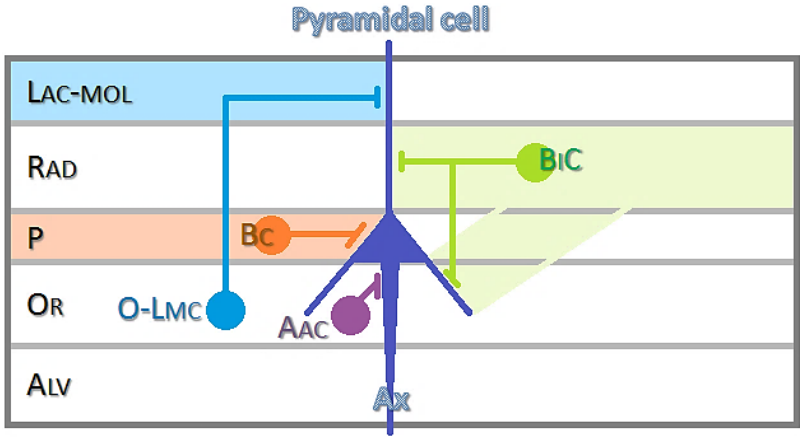


Figure 12 - Interneurons intrinsic connections in Ammon's Horn hippocampal regions. Filled circles represent interneuron's cell bodies with departing coloured lines corresponding to projection sites upon a pyramidal cell. Coloured zones show postsynaptic domains innervated by colour-matching interneurons. BC, basket cells; P, stratum pyramidale; RAD, stratum radiatum; BiC, Bistratified cells; OR, stratum oriens; O-LMC, Oriens lacunosum moleculare cells; LAC-MOL, stratum lacunosum moleculare; AAC, Axo-axonic cells; ALV, stratum alveus. Adapted from Hammond, C. (2015)[11]

Similarly to pyramidal neurons, in-utero electroporation experiments have shown that tangential migration of interneurons is modulated by a paracrine action of glutamate acting through α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors. Furthermore, interneurons reach the presumptive hippocampus at E15[74]. By E16, a superficial migratory stream reaches CA3, whereas the deep stream reaches CA1 but stops at the border of CA3. Interneurons reach the dentate gyrus primordium via the superficial stream by E17[75]. The functional and morphological maturation of hippocampal interneurons then occurs during a protracted period spanning from foetal to postnatal development, resulting in heterogeneous profiles of maturation among the interneurons population. Consequently, at birth, interneurons at different stages of development were shown to be mixed within the same layer: silent interneurons, showing no spontaneous or evoked synaptic currents; interneurons presenting only $GABA_A$ synapses; and interneurons having both GABA and glutamatergic synapses[65]. Then, interneurons follow the same sequence than pyramidal neurons, but it occurs earlier. Moreover, this showed that the majority of pre-natal

interneurons synapses was already active, whereas almost the full population of pyramidal neurons was still quiescent (figure 10.B.)[65].

4) Neurotransmission

The activation of neurotransmitter receptors triggers the modification of ionic conductances through the neuronal membrane. The synaptically-induced opening of channel receptors produces fast ions influxes or effluxes through the cell membrane, transiently dissipating the electrochemical gradients maintained in the cells as well as the membrane resting potential. An entry of positively charged ions (ex: Na^+) or an exit of negative charged ions (ex: Cl^-) will decrease the membrane potential difference and therefore induce a post-synaptic depolarising potential. If the membrane potential reaches the cell's threshold for action potentials, the cell fires. By opposition, an entry of negatively charged ions (ex: Cl^-) or an exit of positively charged ions (ex: K^+) will induce a hyperpolarisation of the membrane potential and induce a post-synaptic inhibitory potential[11].

Glutamate

In the adult CNS, glutamate is the major excitatory neurotransmitter, and GABA the major inhibitory one. As mentioned in the previous section, glutamatergic synapses establishment occurs later than for GABA. In the hippocampus, glutamate acts on two types of receptors: metabotropic receptors (mGluRs) intracellularly coupled to GTP-binding proteins (G proteins), or ionotropic receptors corresponding to ligand-gated cation channels, named according to their preferential agonist (Table 5). Furthermore, glutamatergic excitatory transmission also follows a progressive maturation, with an earlier participation of N-methyl-D-aspartate (NMDA) compared to AMPA receptors[76,77], followed by kainate[78].

GABA

The neurotransmitter GABA is generated in interneurons through enzymatic decarboxylation of glutamate by the enzyme glutamate-decarboxylase (GAD)[45]. GABA released by presynaptic terminals can activate ionotropic postsynaptic $GABA_A$ receptors ($GABA_AR$) principally permeable to chloride (Cl^-) and to a lesser extent to bicarbonate (HCO_3^-) ions[79], and pre or postsynaptic metabotropic $GABA_B$ receptors coupled to $G\alpha_{i/o}$ proteins[11]. GABAergic signaling through $GABA_AR$ depends on the intracellular concentration of chloride ($[Cl^-]_i$), leading in certain conditions to depolarising and even excitatory effects[80]. Importantly, the chloride

electrochemical gradient follows a developmental profile ensueing a depolarising action following $GABA_AR$ activation in the immature CNS, and hyperpolarising action in the mature CNS[80–82]. Interestingly, several pathological conditions have been shown to present increased $[Cl^-]_i$ leading to depolarising and excitatory GABA instead of hyperpolarising and inhibitory[83–86].

METABOTROPIC				IONOTROPIC		
RECEPTOR	GROUP	G-PROTEIN COUPLING	SYNAPTIC SITE	RECEPTOR	IONIC PERMEABILITY	SYNAPTIC SITE
mGluR1 mGluR5	1	$G_{\alpha q}$ Activates phospholipase C	Post synaptic	NMDA (N-methyl-D-aspartate)	Na^+, K^+, Ca^{2+}	Pre and post synaptic
mGluR2 mGluR3	2	$G_{\alpha i}$ Inhibits adenyl-cyclase	Pre synaptic	AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate)	Mainly Na^+, K^+ Sometimes Na^+, K^+, Ca^{2+}	Mostly post synaptic
mGluR4 mGluR6 mGluR7 mGluR8	3			Kainate	Mainly Na^+, K^+ Sometimes Na^+, K^+, Ca^{2+}	Pre and post synaptic

Table 5 - Glutamatergic neurotransmission exerts excitatory effects through two types of receptors: metabotropic and ionotropic. Both types of receptors can be found either on the pre- or post-synapse. Ionotropic receptors for glutamate are cationic ion channels of which calcium (Ca^{2+}) permeability varies on specific expression of particular receptors subunits[11,87].

Developmental regulation of $[Cl^-]_i$

Cation-chloride-cotransporters (CCCs), are the intrinsic membrane proteins regulating the neuronal $[Cl^-]_i$. In neurons, the two main transporters are the Na^+–K^+–$2Cl^-$ importer NKCC1 and the K^+–Cl^- exporter KCC2. During prenatal development, the importer NKCC1 is highly expressed at the neuron's plasma membrane[88], whereas the exporter KCC2 is poorly present[89]; leading to an accumulation of $[Cl^-]_i$ and to depolarising effects of GABA upon $GABA_AR$ activation (Figure 13)[80]. After birth, $[Cl^-]_i$ progressively decreases with age, notably due to the developmentally up-regulated expression of KCC2, leading to a hyperpolarising GABA action[90,91]. Thus, in the mature CNS, GABA release opens $GABA_AR$ channels and causes a net influx of chloride into neurons with consequent membrane hyperpolarisation and reduction of cell firing.

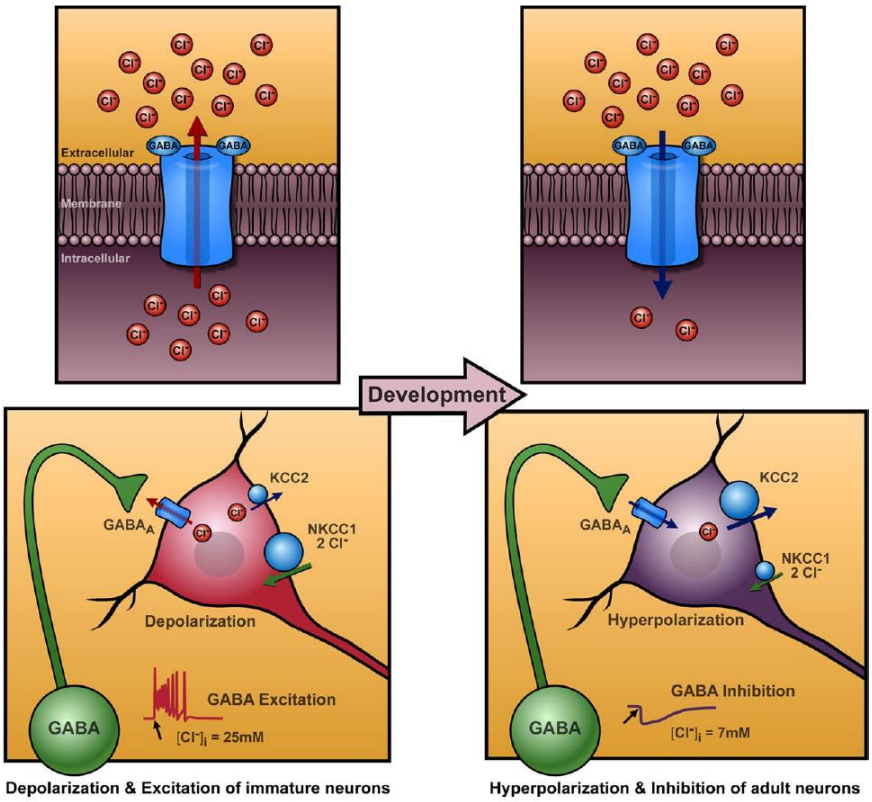


Figure 13 - Neuronal regulation of $[Cl^-]_i$ by CCCs during development and consequences on GABA action. On the left hand, during early development, high expression of the NKCC1 importer and low expression of the KCC2 exporter maintains high $[Cl^-]_i$, inducing outward Cl^- currents upon $GABA_AR$ activation. This resulting depolarisation can lead to an excitatory action of GABA. As the development proceeds, the upregulation of KCC2 and concomitant downregulation of NKCC1 reduces $[Cl^-]_i$. The activation of $GABA_AR$ thus produces inward Cl^- currents, and neuronal hyperpolarisation leading to an inhibitory action of GABA. Adapted from Ben-Ari, Y. *et al* (2007)[80].

5) Hippocampal primitive oscillations

Neuronal activities shape and tune neuronal networks at every stage of development. Immature systems present spontaneous endogenous activities in many animal species and within different regions of the CNS[92,93]. These activities are slow or rhythmic, and are associated to a rise of intracellular calcium concentrations ($[Ca^{2+}]_i$). In addition, they are present at critical periods of development in which peaks of axonal and dendritic growth or synaptogenesis occur and coincide with the period in which GABA is still immature and exerts depolarising actions[94]. Functionally, these immature patterns of activity allow for the progressive transition from a silent CNS to a highly connected one, in which neuronal ensembles communicate by the means of synaptic activities and generate behaviourally-relevant oscillatory patterns.

Calcium spikes

Calcium spikes are intrinsically-generated events occurring at late embryonic stages in mice (E16-19), prior to synapse formation. Their generation requires voltage-gated sodium and L-type calcium channels. These events are brief, uncorrelated, and do not propagate to other neurons[95]. Spontaneous calcium spikes activities occur in various CNS structures such as the neocortical ventricular zone[96], hippocampus[95], spinal cord[97], cortex[98] and striatum[99].

Synchronous Plateaus Assemblies

SPAs are intrinsic non-synapse driven synchronised calcium currents lasting several seconds, 'calcium plateaus', generated by small ensembles of neurons connected by GAP junctions[95]. In the cortex and hippocampus, SPAs appear shortly before birth in mice, peak on the day of birth, and start to decline within the first postnatal days[95,98] (Figure 14). This coherent calcium activity is produced by lasting recurrent burst discharges dependent of voltage dependent sodium and L-Type calcium channels, and the hyperpolarisation activated cationic current I_h. Furthermore, the application of the parturition hormone oxytocin on brain slices was shown to increase the population of SPA cells in hippocampal brain slices. This effect was also produced by bath application of the specific NKCC1 antagonist bumetanide[95]. Therefore, SPAs are a coherent non-synaptic activity pattern specifically enhanced at birth, that are dependent on oxytocin $[Cl^-]_i$ and GABA.

Giant Depolarising Potentials

GDPs correspond to the first spontaneous synaptic-driven pattern of activity in the rat and mouse hippocampus, but also in other species such as the rabbit and the Rhesus monkey[94,100]. They are characterised by recurrent synchronised population discharges occurring at a relatively low frequency (0.1 Hz) mainly originating from CA3 and propagating to CA1. Originally, GDPs were described in the CA3 hippocampal region, but they also occur in CA1 and in granular cells of the dentate gyrus at a lower frequency. However, the septal pole of CA3 expresses the highest pacemaker activity[101]. GDPs frequency increase with the decline of SPAs activity in the early postnatal life, and disappear by the end of the second postnatal week[95]. The generation of GDPs is dependent of the combined depolarising action of GABA and glutamatergic input through NMDA receptors[100,102]. In addition, fate mapping techniques have recently shown that their generation relies on the drive provided by early-born, hyper-connected "hub-neurons" with large arborisation orchestrating their occurrence within the entire network[103–105].

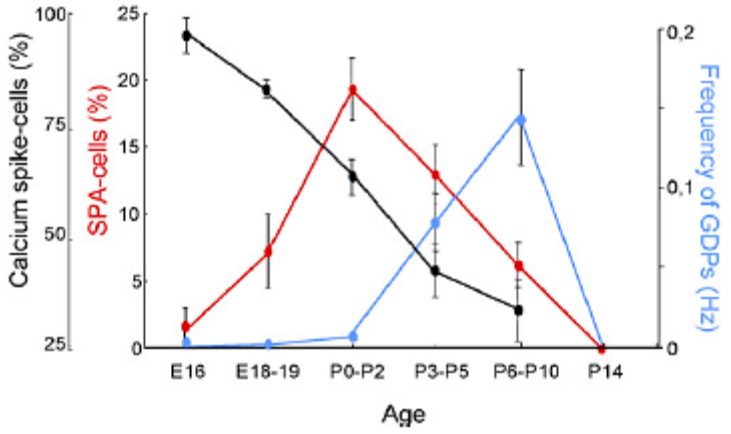


Figure 14 – Maturation of primitive patterns of activity in the hippocampus. Calcium spikes are the first patterns of activity in the developing brain. SPAs appear a couple of days before birth, and peak at P0. GDPs arise following birth, increase as SPAs activities decline, and disappear by P14**.** Adapted from Crépel *et al* (2007)[95].

III – Maternal Immune Activation: MIA

1) History of a risk factor

Neurodevelopmental disorders illustrate the particular vulnerability of the developing central nervous system (CNS) to perturbation. Activation of the maternal immune system by external pathogens, autoimmune disease or inflammatory disorders during pregnancy correspond to the MIA risk factor during early development.

The first reports of the disruptive potential of maternal infection during pregnancy on the child's neurodevelopment were published consecutively to the 1960's rubella epidemic affecting the U.S.. In three different cities (Philadelphia, New-York, and Houston) three research groups reported the teratogenic effects of in-utero exposure to maternal infection in born infants[106]. Among the estimated 1-1.4% of births affected by rubella in Philadelphia, Janet Lindquist and peers reported a spectrum of malformations and defects produced by the congenital infection in infants, designated as Congenital Rubella Syndrome (CRS). This comprised heart malformations, low birth-weight, defective bones calcification, eye cataracts and other defects[107]. In New-York, the American child psychiatrist Stella Chess highlighted a causative link between in-utero rubella infection and the development of autistic psychopathology in children. Among a cohort of 243 preschool children who had been

affected by rubella in utero, 18 cases of autism were identified[108]. Finally, in Houston, the paediatrician Murdina MacFarquahar Desmond and colleagues reported 12.5% children affected by autism among 100 infants showing congenital rubella[106,109].

A similar wave of publications raising concerns between maternal influenza infection and higher schizophrenia rates in the offspring occurred following the 1957 influenza epidemic. Positive correlation between maternal exposure to influenza and increased schizophrenia risks were reported in Finland[110], the United-Kingdom[111], Japan[112], and Australia[113].

More recently, a massive population study comprising all children born in Denmark between January 1st, 1980, and December 31st, 2005 (1,612,342 children) confronted the history of maternal hospital admission caused by infection during pregnancy and the ASD diagnoses reported in the Danish Psychiatric Central Register[114]. This showed that a bacterial infection during the second trimester of pregnancy or a viral infection in the first trimester was correlated with the diagnosis of autism spectrum disorders in children[114].

Epidemiological studies have since then provided a wide coverage of populations exposed to a similar risk factor: maternal infection during pregnancy. As a whole, their convergence towards similar outcomes gave the clue that such infections were able to produce developmental changes ultimately increasing the risk for neurodevelopmental disorders[115–118]. Particularly, two canonical neurodevelopmental disorders were shown to be at risk: schizophrenia and autism.

Autism spectrum disorders (ASDs) are a type of pervasive developmental disorder manifesting before the age of three years. Behaviourally, ASDs are characterised by three core symptoms: abnormal reciprocal social interaction, abnormal communication, and restricted, stereotyped or repetitive behaviour[119].

Schizophrenic disorders typically manifest in adolescence or early adulthood. Schizophrenia symptoms can be of two types: positive and negative symptoms. Positive symptoms are characterised in general by distortions or exaggerations of thinking and perception giving rise to delusions, hallucinations, disorganised speech or inappropriate behaviours. Negative symptoms however, reflect a loss of normal function that can manifest through loss or impoverishment of speech, decreased capacity to express emotions, to experience pleasure or to realise goal-directed behaviours, and deficits of attention[45,119].

2) How the maternal infection risk factor of neurodevelopmental disorders started to be deciphered

In line with the epidemiological reports, research groups started to design animal models of infection during pregnancy and succeeded in inducing altered behavioural outcomes in offspring[120].

A considerable advance in the understanding of the underlying mechanisms induced following maternal infection was brought by the team of late Paul Patterson. In 2003, Patterson's team noticed that either infusing pregnant mice with influenza virus, or inducing a maternal immune response by the means of a viral mimic (the Poly(I:C)), produced similar behavioural impairment of the pre-pulse inhibition in the offspring[121]. In follow-up experiments, they found no signal of virus mRNA in foetal brains, and only rarely in maternal placentae. This demonstrated that the virus did not produce deleterious effects on the foetal brain by directly infecting the foetus, and the authors concluded that the maternal immune activation (MIA) itself would rather be the cause[122]. The final answer to the hypothesis that MIA leads to neurodevelopmental disorders, and hence altered behaviours was given in 2012, when Patterson's team further investigated the behavioural alterations of offspring born after Poly(I:C) induced MIA: eliciting a MIA in the absence of viral infection resulted in offspring displaying behavioural impairments on the three core symptoms of autism[123].

Current animal models of MIA use the same scheme: a component chosen for its immunogenicity is injected at a defined dose to a time-pregnant animal, and the consequences are studied in the offspring (a summary of these models is presented on section 4, Table 5). The two most widely used components to induce a maternal immune response are:

- **Lipopolysaccharide (LPS)**, corresponding to Gram-negative bacterial wall glycolipids. LPS mimics a bacterial infection via binding to the pattern recognition receptor Toll-like receptor (TLR) 4[124], which induces the intracellular activation of the nuclear factor-kappa B (NF-κB) involved in inflammatory responses[125].
- **Polyinosinic-Polycytidylic Acid (Poly(I:C))**, a synthetic double-stranded RNA. Because most viruses produce double-stranded RNA at some point during their replication[126], this component is commonly used as a viral mimetic. It is recognised by TLR3 essentially expressed intracellularly, that triggers the activation of NF-κB and the

production of type I interferons[127]. In the CNS, TLR3 is highly expressed intracellularly by both microglia and astrocytes[128].

However, other studies have induced maternal immune activation by direct infection with either bacteria, viruses, or interleukins[129].

3) Immune mediators of MIA

These components, by triggering a maternal immune response, lead to the release of pro- and anti-inflammatory factors. As a matter of fact, these molecules are the secondary messengers mediating the effects on the brain of the primary component used to trigger the MIA. Because my thesis investigates the effects produced by the viral mimic Poly(I:C), the following section will mainly discuss data that are relevant to this model.

Importantly, the origin of secreted immune factors consequently to MIA can come from different sources. Thus, first trimester human placentae were harvested, and trophoblasts cells cultured to study the immune response induced by Poly(I:C) or LPS stimulation[130]. This showed that after stimulation with Poly(I:C), trophoblast cells rapidly produce Interferon-β (IFNβ) in a dose-dependent manner, with maximal increase of IFNβ secretion observed 48h after treatment and maintained high at 72h. Because the production of type I interferons is characteristic of an immune response to viral products following TLR3 activation[131], this response was not found consecutively to LPS stimulation. Another important mediator of the MIA is Interleukin-6 (IL-6). Indeed, several studies showed the capability of this interleukin to alter the brain transcriptome and induce autistic-like behaviours in the adult offspring when injected alone and prevented when maternal Poly(I:C) injection was coupled to the administration of an IL-6 antibody[132]. Furthermore, a similar abolishment of the MIA-induced behavioural outcomes occurred when MIA was induced in IL-6 knock-out mice[132], or in females in which the receptor for IL-6 (IL-6Ra) was depleted in placental trophoblasts[133]. Actually, activated decidual leukocytes may be the source of MIA-induced placental IL-6, and the site of foetal cells activation by maternal IL-6 in the spongiotrophoblast layer of the placenta[134]. Additionally, increased mRNA expression of tumour-necrosis factor α (TNFα) and IL-1β, and trends for increased expression for IL-17 and IL-10 have also been shown in MIA mouse placenta[134].

Importantly, maternal Il-17 upon activation after MIA has been shown to induce an abnormal cortical phenotype in foetal and adult mice brains, and autistic behaviours in MIA offspring[135]. More precisely, the cortical phenotype comprised abnormal lamination, as well as dysplastic cortical patches that were mainly localised in the primary somatosensory cortex[136,137]. Strikingly, the stimulation of pyramidal cells within these dysplastic patches triggered abnormal behaviours in MIA mice, allowed to discriminate the brain areas targeted by axonal projections of neurons located in the dysplastic patches, and showed that this was responsible for the abnormal behaviours seen in MIA offspring[136]. Thus, in-vivo inhibition of neurons expressing an inhibitory halorhodopsin in the preferential area for dysplasia restored behaviours to control levels[136]. Moreover, the induction of Poly(I:C) MIA in pregnant mice lacking a specific bacteria strain in their gut microbiota responsible for IL-17 secretion, the segmented filamentous bacteria (SFB), failed to reproduce the cortical abnormalities and autistic behaviours, suggesting the maternal microbiota to be an important mediator of MIA-induced immune response[137].

Although no change in microglial cells number and density was shown in Poly(I:C)-MIA foetal brains[138139], microglial cells were shown to respond to MIA through the protein or mRNA expression of immune mediators. More precisely, analysis of microglial cells from E16.5 mouse foetal forebrains following Poly(I:C) MIA at E12.5 showed increased IL-6 mRNA, and increased protein levels for IL-1α, IL-4, and IL-9 but not IL-6[140]. In addition, direct injection of Poly(I:C) to adult mice (i.e. in a non-MIA context) has been reported to stimulate a microglial and astrocytic secretion of IFN-β mediating an enhanced spontaneous activity in the CA1 pyramidal layer of the hippocampus[141].

In addition, in mice, the effect of MIA was also reported on the expression of cytokines in the blood and three brain regions (frontal cortex, cingulate cortex, and hippocampus) across ages spanning from birth to P60. This work showed that (1) most of the 23 cytokines tested were expressed in the serum and brain throughout postnatal development with age and region-dependent variations, and (2) inducing an MIA with Poly(I:C) at E12.5 alters these expression profiles for many of these cytokines in an age and region-specific manner[138].

Then, MIA triggers an acute immune response, and induces long-lasting perturbations of brain intrinsic immune factors, both of which can potently affect brain physiology.

4) Consequences of MIA on the offspring's behaviour and brain physiology

A summary of the behavioural and brain alterations described in several animal models of MIA is given in the table hereafter (Table 6).

Species	Treatment	Time of treatment	Behavioural/Physiological findings	Histological findings
Rat[142]	4mg/kg LPS I.P.	E18-E19	NR	P8: Increased GFAP, decreased MBP; altered microglial staining
Rat[143]	10,000EU/kg LPS I.P.	E10.5	NR	Adult: Fewer TH+ neurons and increased microglial staining in substantia nigra
Rat[144]	1mg/kg LPS S.C.	Alternate days throughout pregnancy	PPI deficit corrected by antipsychotic drugs	Adult: Increased GFAP, MHCII staining of microglia; TH increase in NAC
Rat[145]	2mg/kg LPS S.C.	Daily through pregnancy	PPI deficit corrected by antipsychotic drugs	Adult: DA increased in NAC; DARPP-32 deficit in PFC; increased synaptophysin in PFC and hippocampus
Rat[146]	20–80µg/kg LPS S.C.	E15–E19 (increasing dose schedule)	Increased entries into all arms of plus maze, slips in beam walking test.	NR
Rat[147]	50µg/kg LPS I.P.	E18 and E19	Increased amph-induced locomotion, acoustic startle response	NR
Rat[148]	0.75–1.0 µg/kg Poly(I:C) I.P.	E15 and E16 or E18 and E19	No deficit in PPI	NR
Rat[149]	1mg/kg LPS I.P.	E18	NR	Less MBP, PLP and myelin staining at P9-30; more microglia at E20
Mouse[150]	0.12mg/kg LPS I.P.	E17	Normal exploration and motor function, mostly normal learning/memory but specific deficits	Adult: Smaller, denser neurons in hippocampus; more pyknotic cells in cortex
Mouse[151]	Intranasal influenza	E9	PPI, open field, novel object, social interaction deficit	Large adult brain; pyramidal cell atrophy; Purkinje cell deficit
Mouse[132,152]	20mg/kg Poly(I:C) I.P.	E12	PPI, LI, open field, social interaction deficit	P11 and adult: Purkinje cell deficit
Rat[153]	4mg/kg Poly(I:C) I.V.	E15	LI deficit, enhanced reversal learning, normal water maze, increased amph and MK-801 locomotion	Adult: Pyknotic cells in hippocampus; increased KCl^--stimulated dopamine release in striatum $GABA_A$
Mouse[154–157]	5mg/kg Poly(I:C) I.V.	E9	PPI, LI, open field, working memory deficits; increased amph induced locomotion	$GABA_A$ receptor increase, no increase in pyknotic cells in hippocampus; reduced RLN^+ and PV^+ cells in PFC; reduced D1 and D2 receptors in PFC; enhanced TH in striatum
Mouse[158]	5mg/kg Poly(I:C) I.P.	Daily E12–E17	PPI, open field, working memory deficit, increased amph locomotion	Adult: Altered dopamine metabolism in striatum
Rat[159]	4mg/kg I.V.	E15	PPI deficit	NR
Mouse[160]	20mg/kg Poly(I:C) I.P.	E12	Impaired extinction of conditioned eyeblink response	NR
Mouse[161]	20mg/kg Poly(I:C) I.P.	E12	Adult: Normal LTP at Schaffer collateral synapses; reduced	NR

			frequency and increased amplitude of mEPSCs with increased sensitivity to DA	
Rhesus monkey[162]	0.25 mg/kg Poly(I:C) stabilised with poly-L-lysine	Late 1st trimester and late 2nd trimester	10, 17, 22 months: increased repetitive behaviours; 17, 22, 24 months: decreased affiliative vocalisations	NR
Mouse[163]	500 µg/kg LPS I.P.	E19	P12 to P25: Impaired eIPSCs, decreased sIPSCs and mIPSCs frequency in CA1; Impaired LTD restored by tiagabine and abnormal LTP after ppulse-LFS prevented by tiagabine and $GABA_B$-R antagonist at Schaffer collateral synapses	P18: decreased GAD67 in CA3
Rat[164]	9 µg/kg IL-6 I.P.	E8, E10, and E12 (early exp.: EIL) Or E16, E18, and E20 (late exp.: LIL)	Impaired spatial learning in Morris water maze and increased thigmotaxis	24 weeks: uneven distribution of Nissl substance in EIL-6 rats, neuronal loss in hilus in LIL-rats; increased astroglial network in both. Decreased neuronal density in CA1, CA3 and hilus in females LIL; in CA1 and hilus in females EIL; in CA2, CA3, and hilus in males EIL and LIL. Increased NR1 mRNA in males and females LIL and in females EIL 4 weeks: increased NR1 mRNA in females LIL

Table 6 – Summary of behavioural, physiological and histological findings in models of maternal immune activation. E, embryonic age; NR, Not reported; Amph, amphetamine; DA, dopamine; GABA, g-aminobutyric acid; GFAP, glial fibrillary acidic protein; I.P, intraperitoneal; S.C., subcutaneous; I.V., intravenous; MBP,myelin basic protein; NAC, nucleus accumbens; N.R., not reported; PFC, prefrontal cortex; PLP, proteolipid protein; PPI, prepulse inhibition; LI, latent inhibition; LPS, lipopolysaccharide; TH, tyrosine hydroxylase; RLN, reelin; PV, parvalbumin; LTP, long-term potentiation; LTD, long-term depression; eIPSCs, evoked inhibitory post-synaptic currents; sIPSCs, spontaneous inhibitory post-synaptic currents; mIPSCs, miniature inhibitory post-synaptic currents; ppulse-LFS, pre-pulse low frequency stimulation; exp, exposure. Adapted from Patterson, P.H.(2009)[120]

Epidemiological studies have shown that maternal immune disturbances in the form of infections or inflammatory diseases during pregnancy increase the risk for neurodevelopmental disorders. Accordingly, research work done on MIA rodent models reported abnormal behaviours in the offspring as well as various brain alterations.

IV – Birth is a critical period in development

Birth is a particularly complex biological process in mammals. In a short period of time, lung, gut, cardiovascular, brain, metabolic and endocrine adaptations must take place to allow the adaptation and survival of the foetus in its new environment.

In Humans, parturition is initiated by the rupture of the protective foetal membranes and the release of active molecules comprising progesterone, oestrogen, prostaglandins and corticotropin-releasing hormone (CRH)[165]. CRH release consequently activates the foetal hypothalamic-pituitary-adrenal axis (HPA), a major neuroendocrine system whose activation results in the synthesis of many hormones including cortisol, catecholamines and thyroid hormones[165]. In particular, catecholamines, comprising norepinephrine, epinephrine and dopamine are released by the foetus' HPA axis, producing a plasmatic surge reaching concentrations singularly higher than in resting adults[166]. This surge is responsible for vital adaptive responses, notably for the adaptation of the foetal blood pressure needed to survive to anoxia when passing through the birth canal and increase blood pressure after delivery. Catecholamines also trigger the initiation of thermogenesis needed to maintain the baby's body temperature once exposed to the colder external environment[167,168], and stimulates the initiation of surfactant secretion by pneumocytes to help for the transition to air breathing[169]. At birth, microbial colonisation of the gastrointestinal tract by exposure to maternal vaginal, faecal and skin microbiota is also fundamental for further development of the metabolic, immune, and gastrointestinal systems of the baby[170,171].

Of particular importance, is the implication of cytokines and inflammatory factors in the initiation of delivery. In Humans, in the 3rd trimester, the levels of IL-1β and IL-8 increase at the maternal-foetal interface, in the amnion, chorio-decidua and myometrium[172]. The levels of IL-1β, IL-6, and IL-8 and its receptors are also increased in the lower uterine segment during term Human parturition, where they induce the entry of calcium into myometrial smooth muscle. This increases myometrial smooth muscle contractility and stimulates prostaglandins synthesis through the activation of NF-kB[173,174]. The human uterine tissue also contains macrophages reaching their highest concentration in late pregnancy, responsible for the secretion of IL-1β, IL-6 and TNF-α. Pro-inflammatory cytokine mRNAs are also most expressed in the decidua in Human late gestation, their levels increasing with labour[172]. Then, parturition itself is an acute localised inflammatory process[174].

To avoid deleterious effects of the stressful event that is birth, several neuroprotective mechanisms operate early on, or during parturition.

The foetal brain overcomes a rapid growth and development during the perinatal period, resulting in the development of brain gyrification[167]. However, a regulation of brain growth

takes place in the preparation for delivery, suggested by the fact that the most important phase of this growth has been shown to occur postnatally[175] and a pre-natal slow-down of brain growth has been estimated in infants born at term with appropriate body weight[176].

Furthermore, neuroprotective effects are exerted at birth by supraspinal analgesia mediated by noradrenaline activation of α_2-adrenergic receptors[177], and increasing levels of OT during delivery. This analgesic effect of OT was shown to rely on the reduction in depolarising action of GABA in nociceptive neurons at birth[178]. Similarly, the oxytocin rise was shown to decrease pyramidal cells excitability at birth by transiently reducing $[Cl^-]_i$ in hippocampal neurons, provoking a switch of GABA action from depolarising to hyperpolarising[179]. Importantly, the abolishment of this natural switch was reported in two rodent models of autism, and its restoration by administration of the NKCC1 chloride importer antagonist bumetanide rescued the autistic behavioural phenotypes in offspring[180]. Thus, the regulation of $[Cl^-]_i$ through the neuroprotective effects of oxytocin appears of crucial importance for the prevention of the pathophysiology of neurodevelopmental disorders such as autism.

V – Conclusion

The CNS development is characterised by crucial patterns of events allowing for the genesis of a complex substrate. The morphological and physiological developmental features presented in the developing central nervous are representative of the advancement degree in the building process. Disruption of developmental processes by risk factors have been shown to produce alterations in the course of the naturally occurring events, resulting in lasting pathological consequences (figure 15)[181–185]. In my thesis, the working hypothesis is that a early developmental risk factor such as Maternal Immune Activation during pregnancy, which leads to the development of neurodevelopmental disorders such as ASDs, affects the morphological and physiological development of hippocampal neurons, and leads to the disruption of the capital neuroprotective GABA switch occurring at birth.

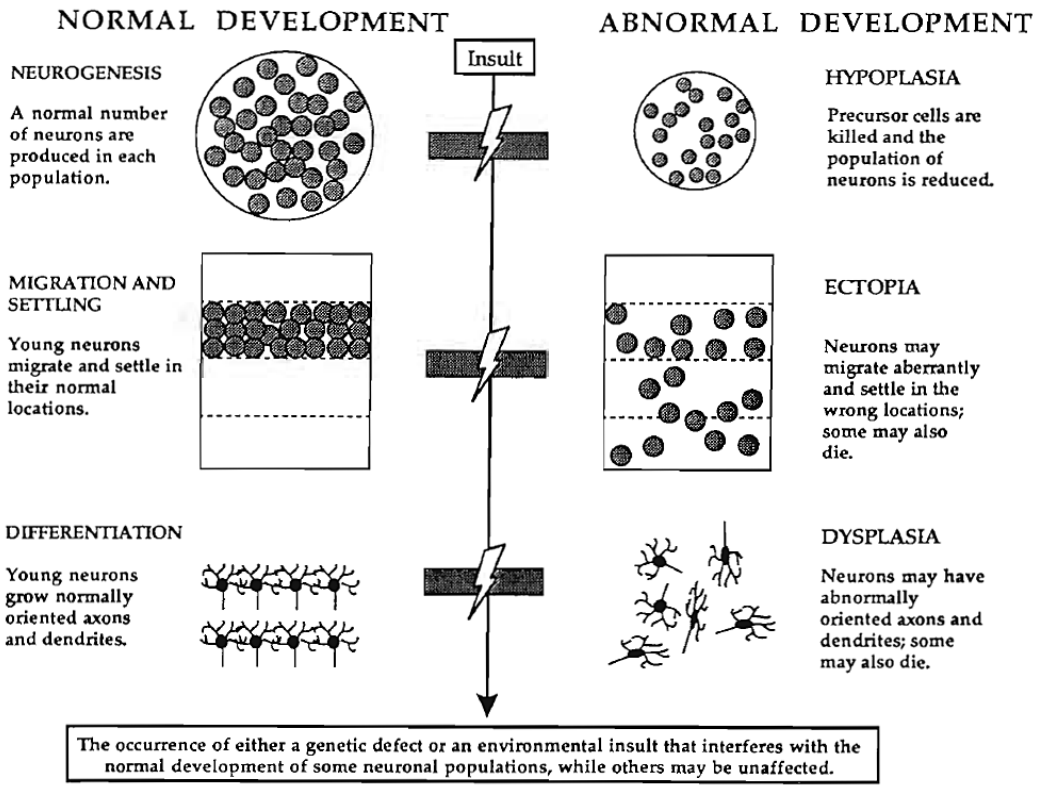


Figure 15 – Depiction of diverse effects produced by insults at different stages of the neuronal development. From Bayer S.A. *et al.* (1993)[186].

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The GABA developmental shift is abolished by Maternal Immune Activation already at birth

Full-length article

The GABA developmental shift is abolished by Maternal Immune Activation already at birth

Amandine Fernandez[a,b,c], Damien Guimond[a], Camille Dumon[a,c], Roman Tyzio[a,b,c], Paolo Bonifazi[d], Natalia Lozovaya[a], Nail Burnashev[b,c], Diana C Ferrari[a]*, Yehezkel Ben-Ari[a,b]*

[a] Neurochlore, Ben-Ari Institute of Neuroarcheology (IBEN), Bâtiment Beret-Delaage, Grand Luminy biotech, 163 route de Luminy, Marseille 13288, Cedex 09, France

[b] Mediterranean Institute of Neurobiology (INMED), INSERM UMR1249, Marseille, France

[c] Aix-Marseille University UMR 1249, Marseille, France

[d] Biocruces Health Research Institute, Barakaldo, Spain & IKERBASQUE: The Basque Foundation for Science, Bilbao, Spain

* Corresponding authors.

E-mail addresses: ben-ari@neurochlore.fr (Y.B.-A.), ferrari@neurochlore.fr (D.C.F.)

ABSTRACT

Epidemiological and experimental studies suggest that maternal immune activation (MIA) leads to developmental brain disorders, but whether the pathogenic mechanism impacts neurons already at birth is not known. We now report that MIA abolishes in mice the oxytocin-mediated delivery GABA shift from depolarising to hyperpolarising in CA3 pyramidal neurons, and this is restored by the NKCC1 chloride importer antagonist bumetanide. Furthermore, MIA hippocampal pyramidal neurons at birth have a more exuberant apical arbour organisation and increased apical dendritic length than age-matched controls. The frequency of spontaneous GABAergic postsynaptic currents is also increased in MIA offspring, as well as the pairwise correlation of the synchronised firing of active cells in CA3. These alterations produced by MIA persist, since at P14-15 GABA action remains depolarising, produces excitatory action, and network activity remains elevated with a higher frequency of spontaneous glutamatergic postsynaptic currents. Therefore, the pathogenic actions of MIA include important alterations already at birth.

Keywords: **GABA, birth, Poly(I:C), apical arborisation, excitatory-inhibitory imbalance**

1. Introduction

Epidemiological studies indicate that maternal infection and inflammatory processes during pregnancy are risk factors for developmental disorders in offspring (Atladóttir et al., 2012, 2010; Brown, 2012; Estes and Mcallister, 2016; Knuesel et al., 2014; Lyall et al., 2014; Patterson, 2009, 2002; Zerbo et al., 2016). Specifically, a bacterial infection during the second trimester of pregnancy or a viral infection in the first trimester has been correlated with the diagnosis of autism spectrum disorders (ASD) in children (Atladóttir et al., 2010). To investigate the effects of in-utero exposure of the foetus to maternal immune activation (MIA) various animal models have been designed (Bilbo et al., 2018; Dean et al., 2015; Giovanoli et al., 2015a; Solek et al., 2017). Injection in pregnant rodents of cytokines (Smith et al., 2007), bacterial cell wall endotoxin lipopolysaccharide (LPS) (Custódio et al., 2017; Kirsten and Bernardi, 2017) or the viral mimic double-stranded RNA polyinosinic-polycytidylic acid (Poly(I:C)) generate offspring with a variety of autistic-like behavioural impairments (Hsiao and Patterson, 2011; Meyer et al., 2008; Smith et al., 2007). Indeed, pregnant mice exposed to Poly(I:C) on embryonic day 12.5 (E12.5) deliver offspring that in adulthood display the three core symptoms of autism: reduced sociability, impaired communication skills, and stereotypic behaviours (Malkova et al., 2012). Experimental studies attempting to decipher the

pathophysiological mechanisms triggered by MIA have highlighted the wide diversity of brain areas impacted by the prenatal insult, such as the neocortex (Kim et al., 2017; Paylor et al., 2016; Richetto et al., 2014; Shin Yim et al., 2017), hippocampus (Giovanoli et al., 2015b; Wolff and Bilkey, 2015), and cerebellum (Shi et al., 2009), producing a variety of alterations. Particularly, Poly(I:C)-induced MIA offspring were shown to exhibit pleiotropic neuropathophysiological disturbances. This included a loss of neuronal layer identity with somatosensory cortical disorganised patches (Kim et al., 2017; Shin Yim et al., 2017); a reduction of parvalbumin interneurons' perineural nets (Paylor et al., 2016) and an altered GABAergic (γ-aminobutyric acid (GABA)) transcriptome (Richetto et al., 2014) leading to decreased GAD67 (Glutamate decarboxylase) expression in the prefrontal cortex; and in the hippocampus, synaptic deficits (Giovanoli et al., 2015b) and alterations of place-cell firing (Wolff and Bilkey, 2015). However, whether and how MIA hinders developmental sequences is not known. This is important as perturbation of developmental sequences might deviate their normal course, leading to misplaced/misconnected neurons and aberrant networks, a concept known as Neuroarchaeology (Ben-Ari, 2008). Indeed, neurons with immature features might constitute a leading pathogenic mechanism perturbing relevant brain network oscillations. In keeping with this hypothesis, Corradini and colleagues have recently shown that the developmental GABA excitatory to inhibitory shift is not present in cortical neurons of MIA offspring, leading to paradoxical excitatory actions of GABA in P20 rodents (Corradini et al., 2017). However, whether these alterations are already present at birth is not known.

Birth is a highly complex and stressful event with a high degree of vulnerability (Boksa et al., 2006; Brander et al., 2017; Chudal et al., 2014; Say et al., 2016; Wang et al., 2017). We reported in earlier studies that birth is associated with a neuroprotective oxytocin-mediated abrupt reduction of intracellular chloride ($[Cl^-]_i$) allowing for hyperpolarising actions of GABA (Tyzio et al., 2006). This is abolished in Fragile X (FRX) and intrauterine Valproic Acid (VPA) rodent models of ASD, and the effects are persistent with GABA exciting neurons also in pre-wean (P14-15) juvenile offspring. In addition, maternal administration of the NKCC1 specific antagonist bumetanide shortly before and during delivery restored the hyperpolarising and inhibitory actions of GABA at birth and in pre-wean juvenile offspring, and attenuated behavioural deleterious sequels (Eftekhari et al., 2014; Tyzio et al., 2014). These results suggest that GABA alterations already present at birth could lead to long-term consequences.

We now report that Poly(I:C)-induced MIA alters the excitatory to inhibitory switch of GABA already at birth due to higher $[Cl^-]_i$ levels as attested by measures of the $GABA_AR$ driving force and its restoration by bumetanide. In addition, pyramidal neurons of hippocampal CA3 region at birth present longer and abnormally sinuous apical tree in MIA offspring. Hippocampal network activity is also altered, with increased frequency of GABAergic postsynaptic currents and a higher pairwise correlation of firing onset in CA3 pyramidal neurons. These effects are persistent, with excitatory GABA action at P14-15, and higher frequency of glutamatergic postsynaptic currents leading to a long-lasting inhibitory *vs.* excitatory imbalance in MIA offspring. Altogether, our work shows that Poly(I:C)-induced MIA produces major physiological and morphological changes already at birth.

2. Materials and Methods

2.1. Animals and MIA induction

All animal experiments were carried out according to the guidelines set by the European Communities Council Directive (2010/63/EU). Time-pregnant female C57BL6/J mice at embryonic day 6 to 8 (E6 to E8; Charles River Laboratories) were housed on arrival in an ABSL2 animal-housing facility with *ad libitum* access to food and water under a 12h/12h light/dark cycle. Mice were left undisturbed until the day of MIA induction. Poly(I:C) (20 mg/kg, Polyinosinic-Polycytidylic Acid Potassium Salt, Sigma-Aldrich) or an equivalent volume of sterile saline solution was administered to the pregnant dams at E12 by intraperitoneal injection as described previously (Hsiao and Patterson, 2011). For the injection, Poly(I:C) was dissolved in sterile saline to a final concentration of 5 mg/mL.

2.2. Acute brain slices

Brains from P0 and P14-15 pups were removed and immersed into ice-cold solution containing (in mM): 132.5 choline chloride, 2.5 KCl, 1.23 NaH_2PO_4, 3 $MgCl_2$, 0.7 $CaCl_2$, 25 $NaHCO_3$, 8 glucose. Horizontal hippocampal brain slices (300-400 µm thick) were cut on a vibratome (VT1200 Leica Microsystems). Slices were then left to recover for 1 hour at room temperature (22-25°C) in artificial cerebrospinal fluid (aCSF) with containing (in mM): 126 NaCl, 3.5 KCl, 1.2 $NaHPO_4$, 2 $CaCl_2$, 1.3 $MgCl_2$, 11 glucose, 25 $NaHCO_3$ (pH 7.4). All solutions were bubbled with 95%O_2/5%CO_2.

2.3. Electrophysiology

For electrophysiological recordings of CA3 pyramidal neurons, slices were placed in a conventional fully submerged chamber superfused with aCSF at a rate of 2-3 mL/min at room temperature.

2.3.1. Single-channel voltage-clamp recordings

The driving force of γ-aminobutyric acid type A ($GABA_A$) receptor (DF_{GABA}) was estimated by single $GABA_AR$ channel recordings in cell-attached configuration using an EPC-10 amplifier (HEKA Elektronik Dr. Schulze GmbH). 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). Abnormal NKCC1-dependant intracellular chloride accumulation was tested with acute application of the specific NKCC1 antagonist bumetanide. Briefly, DF_{GABA} was determined on pyramidal cells before and after a 15 minutes bath application of bumetanide (10 µM, Sigma-Aldrich). Recordings were digitized and analysed as described previously (Khazipov et al., 1995; Tyzio, 2003).

2.3.2. Whole-cell recordings

Whole-cell recordings were performed with a Multiclamp 700B amplifier (Molecular Devices) and EPC-10 amplifier (HEKA Elektronik Dr. Schulze GmbH). Data were acquired at 10 kHz and filtered at 2.4 kHz. 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. 0.5-1% biocytin (Sigma-Aldrich) was added to the pipette solution for post-hoc morphological reconstruction at P0 (see below). Pipette resistance was 6-8 MΩ. Spontaneous glutamatergic postsynaptic currents (sGlu PSCs) were recorded for 15 minutes at the reversal potential for GABAergic currents (-70 mV). Spontaneous GABAergic postsynaptic currents (sGABA PSCs) were recorded for 15 minutes at +10 mV where the potential for glutamatergic currents is reversed or negligible. After recording, data were filtered using a Bessel low-pass 500 Hz filter to reduce the root-mean-square (RMS) noise and improve the signal-to-noise ratio, and analysed using Clampfit 10.4 (Molecular Devices) and Mini Analysis 6.0 (Synaptosoft Inc). The following experimental parameters were similar between groups: animal's age, cell depth, number of events analysed for P14-15 sPSCs (400 events), recording duration analysed for P0 sPSCs (5 minutes).

2.3.3. Intrinsic properties

The membrane capacitance (Cm) and resting membrane potential (i.e. membrane potential at I=0, V_{rest}) were measured immediately after establishing the whole-cell configuration. Action potential firing pattern of the cell was recorded at least 5 minutes after the start of the dialysis to allow intracellular medium diffusion into the cell. The holding current (I_h) and the series resistance (R_s) in response to a 5mV pulse were monitored throughout the recording. Criteria for data inclusion for analysis were: $I_h \geq -50pA$ at $V_{hold}=-70mV$, $R_s \leq 25M\Omega$, and R_s change over recording time ≤ 20%. Additionally, cells were included for analysis if their firing pattern was typical of a pyramidal cell. Post-hoc biocytin revelation provided the cells' morphology and location within hippocampal CA3 pyramidal cell layer at P0.

2.3.4. Cell-attached recordings

Cell-attached recordings of action potential firing were performed with a Multiclamp 700B amplifier (Molecular Devices) on P14-15 hippocampal slices using 7-10 MΩ borosilicate glass pipettes filled with aCSF. After a baseline period of at least 10 minutes, isoguvacine (specific $GABA_A$ receptor agonist, 10µM, Sigma-Aldrich) was bath applied for 120-140 seconds and washed for 15 minutes. Baseline spiking frequency was defined as 100% of spiking activity. Spiking frequency during the application of isoguvacine was calculated as a percentage of the baseline spiking frequency. Firing activities were analysed offline with Mini Analysis 6.0 (Synaptosoft Inc). To rule out the possibility of artifactitious spiking variations, only cells showing a maximum difference of 30% spiking from baseline period compared to wash were included in the analysis. The threshold amplitude for detecting action potentials was set at twice the baseline noise.

2.4. Calcium imaging

2.4.1. Recordings

After 1 hour recovery, P0 slices were incubated for 25 to 30 minutes in a lightproof oxygenated chamber heated at 37°C filled with 2.5 mL aCSF with 25 µL Fura-2AM solution containing: 50 µg Fura-2 (Molecular Probes, Life Technologies), 48 µL DMSO (Sigma), 2 µL Pluronic-Acid (Life Technologies). Slices were then left to recover for 30 min to 1 hour before recording under continuously superfused oxygenated aCSF (O_2/CO_2 – 95%/5%; 3 mL/min) heated at 37°C. Slices were imaged using a multi-beam two-photon laser scanning apparatus (Trimscope-LaVision Biotec) coupled to an Olympus microscope (wide numerical aperture 20X, NA 0.95, Olympus) as described previously (Crépel et al., 2007; Dehorter, 2011). Laser scanning was performed with a 475x355 µm scanfield using a CCD camera (LaVision Imager 3QE) with a 4x4 binning, time resolution of 137 ms, and a duration of 1000 frames per movie.

2.4.2. Analysis

Calcium imaging recordings were pre-processed using an upgraded version of the custom designed MATLAB (MathWorks) software "Hippo" previously described (Bonifazi et al., 2009; Crépel et al., 2007) to identify the cell contours, extract the average calcium signals from the detected cell areas, and mark the onsets-offsets of the calcium events.

For each movie, active cells were defined as those exhibiting calcium events with a minimum 5% fluorescence deflection within the recording time. Calcium plateaus were defined as long-lasting events during which fluorescence deflection was sustained for at least 30 frames (Crépel et al., 2007; Ferrari et al., 2012).

To calculate the pairwise correlation between active cells, the calcium signal of each cell was converted into a binary time series preserving the temporal resolution, with the ones marking the time window between onset

and offset of each calcium event. The correlation between time series was calculated according to the following equation:

$$C_{ij} = \frac{\sum_{t=1}^{T} S_i(t) * S_j(t)}{\sqrt{N_i * N_j}}$$

where t is the frame number, S_i and S_j are the time series of cell i and j respectively, T is the total number of frames, N_i and N_j are the total number of ones in the time series of neuron i and j respectively. Note that this is the equivalent of calculating the cosine between the two vectors representing the binary time series.

To compute the correlation between the plateau cells, similarly to what was described in Crépel et al (2007), the binary time series of the event's onsets for the plateau cells was smoothed with a Gaussian (σ = 1 frame). The correlation was then calculated according to the equation 1 and the significance of each correlation value with an accuracy of p<0.001 was estimated from surrogate data sets, in which the events were randomly reshuffled in time.

2.5. Biocytin-filled CA3 pyramidal neurons morphological reconstruction

After whole-cell patch clamp recording, P0 slices were immediately immersed in Antigenfix (DiaPath) for overnight fixation. Slices were then rinsed 3 times for 10 minutes in 1X PBS before being stored at 4°C in 1X-PBS 0.01% Azide until biocytin revelation. For biocytin revelation, slices were first rinsed as described above, incubated in blocking solution (1X-PBS, 0.3% Triton, 5% Normal Goat Serum) at room temperature for one hour under gentle agitation, and incubated overnight at 4°C in Streptavidin-Alexa 555 (1/1000, Thermo Fisher-Invitrogen) in blocking solution under gentle agitation. The following day slices were rinsed as previously, incubated for 10 minutes in 1X PBS- 1/1000 Hoechst (Sigma-Aldrich), and rinsed before mounting (Fluoromount-G™ Slide Mounting Medium, Electron Microscopy Sciences). Neurons were stack-imaged with a Leica SP5-X confocal microscope (Leica Microsystems) at a 400Hz frequency, 1024x1024 pixels wide field, Airy 1 pinhole, and 0.5 µm steps. Neurons were reconstructed on image-stacks using the Fiji software (Schindelin et al., 2012)(open-source platform) semi-automated Plugin Simple Neurite Tracer (Longair et al., 2011). Sholl analysis was done with Fiji. Only cells showing a single apical primary dendrite were considered for morphological analysis.

2.6. Statistics

DF_{GABA} data at P0 and P14-15 were analysed by two-tailed T-test. sGlu and sGABA PSCs at P0 and P14-15, calcium percentage of active cells and plateau cells, morphological metrics and intrinsic pyramidal cell properties were analysed by Mann-Whitney test. For cell-attached P14-15 data, paired T-test was conducted within groups to compare spiking frequency of the same cell before and after application of isoguvacine. Values are indicated as mean±S.E.M. Differences were considered significant when p ≤0.05.

3. Results

3.1. Depolarizing actions of GABA and altered apical dendritic arborisation in CA3 pyramidal neurons at birth after MIA

To test the hypothesis that Poly(I:C)-induced MIA offspring present physiological alterations on the day of birth (postnatal day 0, P0), we evaluated GABAergic activity. We used non-invasive cell-attached $GABA_A$ receptor mediated single-channel current recordings to estimate the driving force of GABA (DF_{GABA}). In CA3 pyramidal neurons from P0 saline-treated animals the DF_{GABA} was slightly hyperpolarising (-1.2±1.1 mV, Fig. 1A and B). In contrast, the DF_{GABA} of P0 MIA offspring was depolarising (9.4±1.5 mV; Two-tailed T-test, p<0.001; Fig. 1A and B). Thus, the birth-associated transient GABA hyperpolarising switch (Tyzio et al., 2006) is abolished in MIA offspring. Bath application of bumetanide (specific antagonist of the NKCC1 chloride importer) decreased intracellular chloride ($[Cl^-]_i$) and the DF_{GABA} in saline-treated (-2.8±0.9 mV; Two-tailed T-test, p<0.001) and MIA-treated (-2.1±1.2 mV; Two-tailed T-test, p<0.001; Fig. 1B) pyramidal neurons, switching in the latter the polarity of GABA from depolarizing to slightly hyperpolarizing. Whole-cell voltage clamp recordings were then performed to determine intrinsic, passive and firing properties. These properties including passive membrane properties, firing and action potential properties were not different in control and MIA treated pups (Supplementary table 1).

Biocytin-filled neurons were post-hoc reconstructed for morphological analysis (Fig. 1C and Supplementary Fig. 1). MIA increased the total apical dendrites length (1210±80.13 µm vs 706.7±89.56 µm; Mann-Whitney Test, p<0.001; Fig. 1D), and the ending radius (136.3±6.30 µm vs 103.9±9.16 µm; Mann-Whitney Test, p<0.005; Fig. 1E) of P0 CA3 pyramidal neurons compared to saline neurons. Furthermore, even if the number of nodes (21.44±3.235 vs 25.67±2.297; Mann-Whitney Test, not significant; Fig. 1F) and critical radius (58.82±8.35 vs 74.23±5.5; Mann-Whitney Test, not significant; Fig. 1G) were not different, Sholl analysis showed altered organisation of MIA apical morphology compared to control neurons (Fig. 1H). Thus, following MIA, neurons have a bigger dendritic arbour at birth.

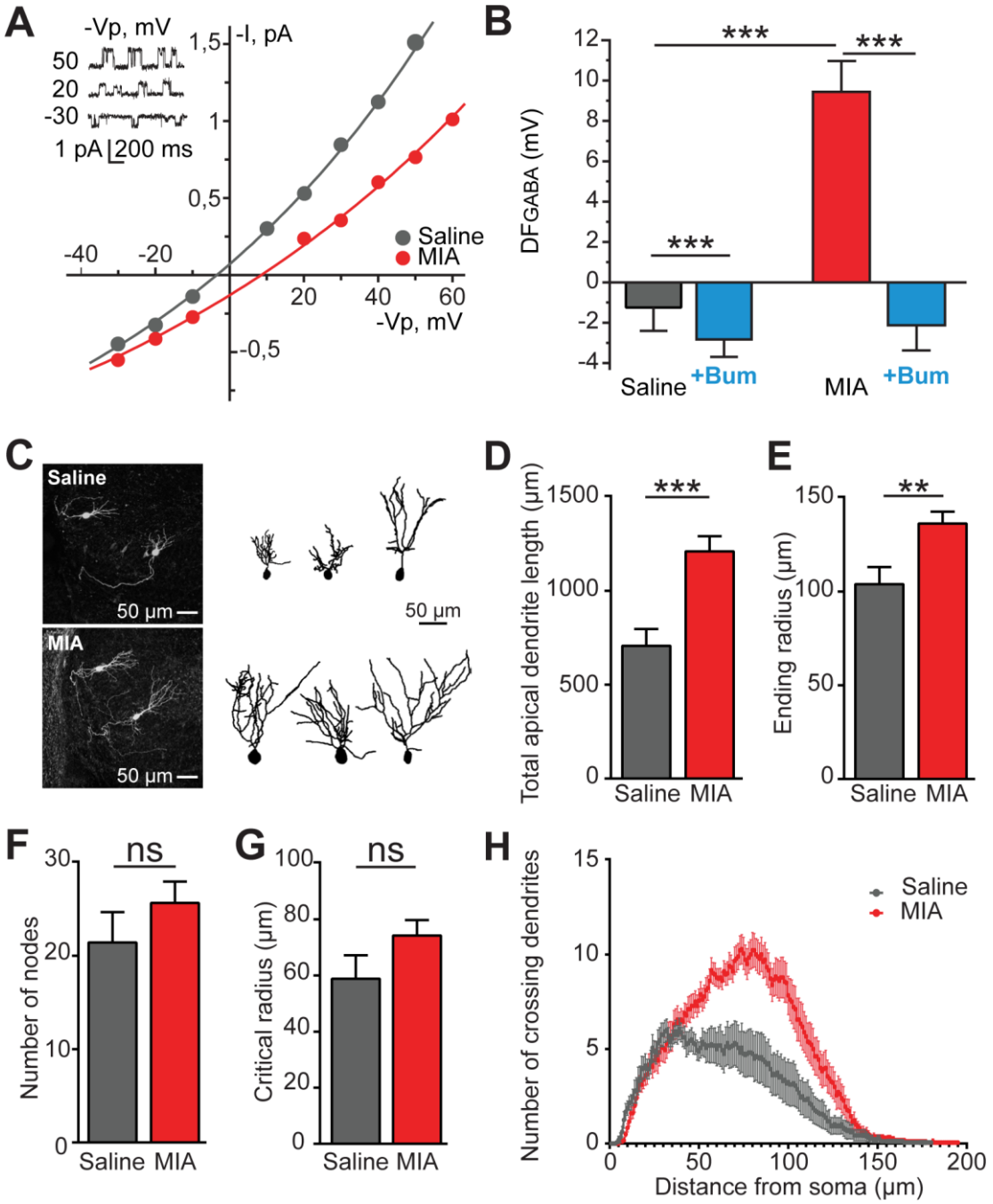

Fig. 1. MIA-induced depolarising GABA action and altered apical dendritic arborisation in CA3 pyramidal neurons at birth. (A) Representative I/V curves of hippocampal CA3 pyramidal cells at P0 using cell-attached recordings of single $GABA_AR$ channels for the estimation of the DF_{GABA}. Each dot shows currents recorded as a function of imposed voltages in a representative P0 saline (grey) and MIA (red) pyramidal cell. Insert shows single $GABA_A$ currents recorded at imposed voltages (-Vp, mV) in a control saline P0 pyramidal cell. (B) Average values of DF_{GABA} in saline (n=51 cells) and MIA (n=52 cells) neurons, with bumetanide action on DF_{GABA} (10 µM, blue; Saline+Bum n=25 cells; MIA+Bum n=39 cells). Two-tailed T-Test, *** p<0.001. (C) Confocal images of biocytin-filled CA3 pyramidal neurons from saline (top) and MIA (bottom) P0 offspring and representative reconstructed apical arbours. (D) The total length of apical dendrites is increased in MIA P0 pyramidal neurons compared to saline. (E) The ending radius of MIA P0 pyramidal neurons is larger than saline controls. (F) The mean number of nodes per radius is similar between control and MIA neurons. (G) The critical radius, at which neurons show the maximum number of intersections, is not significantly different between control and MIA neurons. (H) Sholl analysis illustrating morphological differences of apical dendrites shows altered dendritic arborisation in MIA (red) neurons compared to saline (grey). (D-H) Saline neurons n=16; MIA neurons n=18 cells. Mann-Whitney Test, *** p<0.001, ** p<0.005.

3.2. MIA enhances GABAergic postsynaptic currents and increases pairwise correlation of active cells at birth

We further evaluated GABAergic activity using whole-cell voltage clamp recordings to investigate spontaneous GABAergic post synaptic currents (sGABA PSCs). In P0 CA3 pyramidal neurons of MIA offspring the frequency of sGABA PSCs was close to double of control offspring (0.24±0.03 Hz vs 0.14±0.02Hz; Mann-Whitney Test, p<0.01; Fig. 2A and C). In contrast, the frequency of spontaneous glutamatergic PSCs (sGlu PSCs) at P0 was not different in MIA pyramidal neurons compared to saline ones (0.14±0.03 Hz vs 0.18±0.04 Hz; Mann-Whitney Test, not significant; Fig. 2B and D). Therefore, Poly(I:C)-induced MIA increases selectively GABAergic but not glutamatergic spontaneous activity of CA3 pyramidal neurons at birth.

Hippocampal CA3 primitive oscillations were further investigated in MIA offspring by two-photon calcium imaging of P0 acute brain slices (Fig. 2E1). Semi-automated detection of cells (Fig. 2E2) and evaluation of calcium activity (Fig. 2F) revealed that the percentage of active cells was not different in MIA CA3 networks compared to saline (39.77±3.32% vs 36.41±3.19%; Mann-Whitney Test, not significant; Fig. 2G). However, the hippocampal networks of MIA offspring presented a significantly increased correlation in the calcium events occurrence in active cellular pairs (15 saline networks, 14 MIA networks; Kolmogorov Smirnov test, p <0.001; Fig. 2H). Despite a tendency, no significant difference was found in the percentage of cells presenting a plateau calcium activity (22.72±2.21% vs 16.35±1.76%; Mann-Whitney Test, not significant; Supplementary Fig. 2A), and the distance-dependent pairwise correlation of calcium plateau cells (Kolmogorov-Smirnov, p=0.06, not significant; Supplementary Fig. 2B-E) between groups. Hence, more neurons generate correlated activity at birth in MIA than control hippocampi.

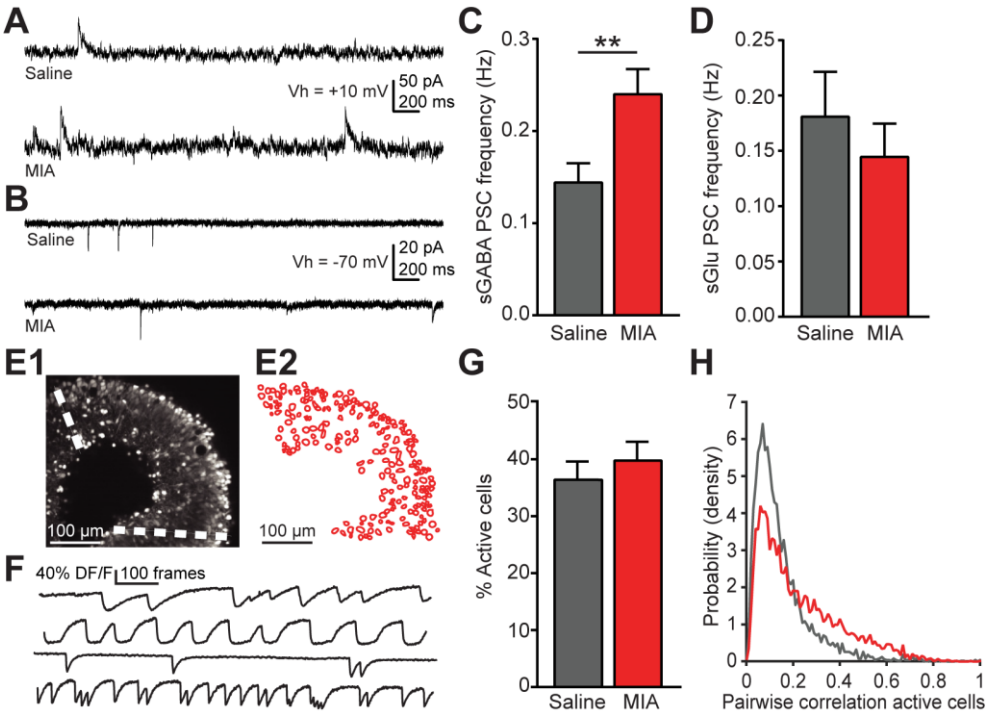

Fig. 2. Enhanced frequency of GABAergic postsynaptic currents and increased pairwise correlation of active cells at birth. (A) Representative traces of sGABA PSCs and (B) sGlu PSCs recorded in whole-cell voltage clamp configuration of P0 pyramidal cells of saline and MIA offspring. (C) The frequency of sGABA PSCs is increased in MIA (n=30 cells) compared to saline (n=31 cells). Mann-Whitney Test, ** p<0.01. (D) The frequency of sGlu PSCs is not different between saline (n=29 cells) and MIA (n=30 cells). (E.1) Representative image of a P0 hippocampal slice loaded with the Fura-2-AM fluorescent calcium sensor and (E.2) corresponding semi-automated contours detection. Dashed lines on picture define the CA3 area in which further steps of analysis were carried out. (F) Representative traces of fluorescence emitted by cells loaded with Fura-2-AM. Each trace corresponds to fluorescence variations emitted by a single cell. (G) The percentage of active cells in P0 CA3 networks is similar in saline (n=15 networks) and MIA conditions (n=14 networks). Mann-Whitney test not-significant. (H) Correlation between the calcium events timing in pairs of active cells. Only pairs showing correlations above zero, i.e. with at least one overlapping event, were pooled and included in the plot. Kolmogorov Smirnov test, p <0.001.

3.3. The depolarising and excitatory actions of GABA are persistent in MIA offspring

We further evaluated if the alterations observed on the day of birth persisted in pre-wean juvenile MIA offspring. The DF_{GABA} continued to be depolarising in MIA pyramidal neurons at P14-15 (11.7±5.1 mV), whereas it was slightly hyperpolarising in control neurons (-3.2±4.1 mV; Two-tailed T-test, p<0.001; Fig. 3A and B). We next assessed if the depolarizing action of GABA was associated with neuronal excitation using the specific $GABA_A$ receptor agonist isoguvacine in cell-attached configuration. As expected, isoguvacine decreased the basal spiking frequency of neurons recorded in control animals (67.24±12.87 % of basal spiking; paired T-Test, p<0.05; Fig. 3C and D), indicating that GABA exerts an inhibitory action on saline neurons. In contrast, isoguvacine increased the basal spiking frequency of MIA neurons (137.1±11.5 %; paired T-Test, p<0.05; Fig. 3C and D), indicative of an excitatory action of GABA at P14-15. Spiking activity was similar before the application of isoguvacine and after washout of the drug in both MIA and saline conditions (Fig. 3D, right). Consequently, MIA induces persistent GABA depolarising and excitatory activities in pyramidal hippocampal neurons of pre-wean juvenile pups.

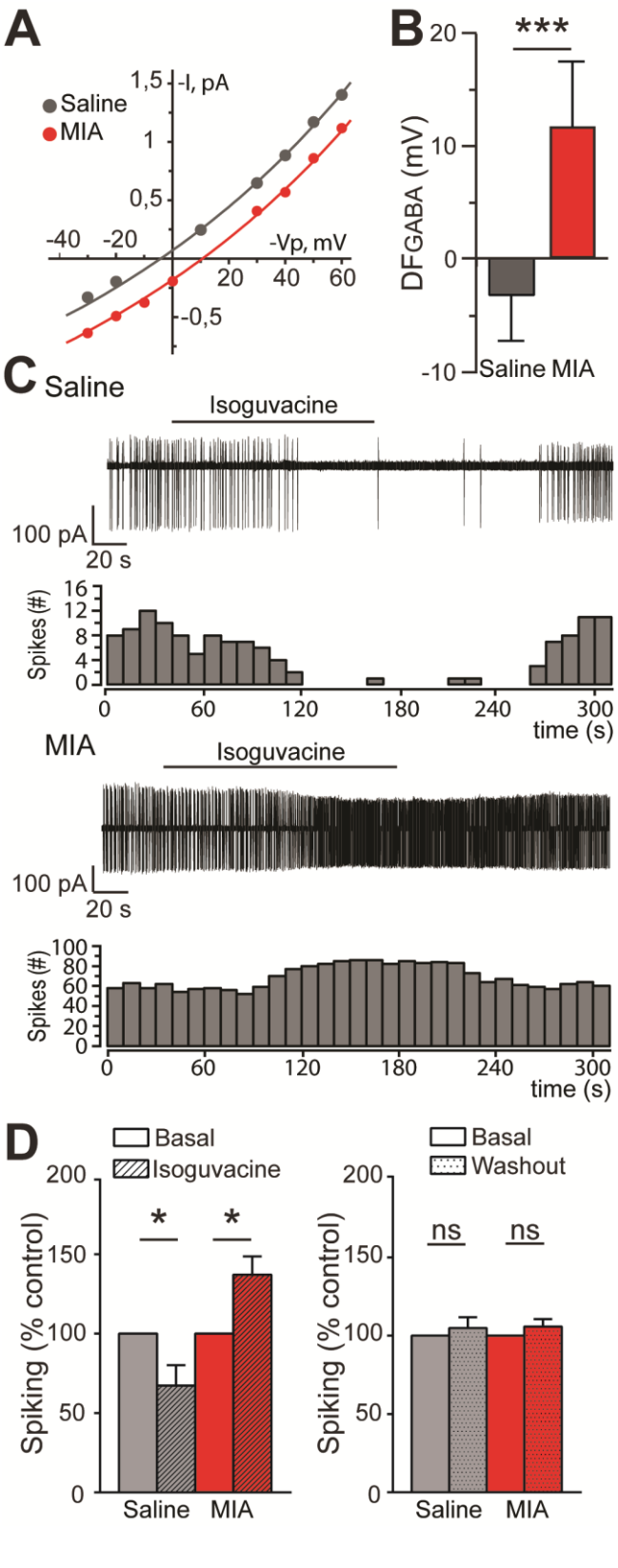

Fig. 3. The driving force of GABA remains depolarising in two-week old MIA offspring, and is accompanied by excitatory GABA action in CA3 pyramidal neurons. (A) Representative I/V curves of P14-15 hippocampal CA3 pyramidal cells recordings of $GABA_AR$ single-channel currents for the estimation of the DF_{GABA} in a representative saline (grey) and MIA (red) pyramidal cell. (B) Average values of DF_{GABA} in saline (n=24 cells) and MIA (n=19 cells) neurons. Two-tailed T-Test *** p< 0.001. (C) Representative traces of P14-15 pyramidal cells spiking activity using cell-attached recordings and associated spike quantification per 10 seconds bin in saline (upper panels) and MIA conditions (bottom panels). Bar above traces indicate time window during which isoguvacine (10µM) was bath applied. (D) Mean spiking frequency of saline (n=9 cells) and MIA (n=9 cells) P14-15 pyramidal neurons upon isoguvacine application (left panel), and during the washout recording period (right panel) were normalised to the basal spiking frequency. Mann-Whitney Test, * p<0.05.

3.4. Glutamatergic but not GABAergic PSCs frequency and amplitude are enhanced in pre-wean MIA offspring

Spontaneous PSC recordings were performed in pyramidal neurons at P14-15 to assess network activity in CA3 after MIA. The frequency and amplitude of sGABA PSCs recorded in MIA offspring neurons (8.88±1.31 Hz; 63.73±3.77 pA, respectively; Fig. 4A and B) were similar to those recorded in neurons of control offspring (6.83±1.48 Hz; 61.77±3.50 pA; Mann-Whitney Test, not significant). In contrast, the frequency of sGlu PSCs was increased in MIA compared to saline neurons (21.45±1.83 Hz vs 13.4±1.54 Hz; Mann-Whitney Test, $p<0.005$; Fig. 4C and D), and so was the amplitude (14.59±0.91 pA vs 11.8±0.69 pA; Mann-Whitney Test, $p<0.05$; Fig. 4C and D). Therefore, MIA enhances sGlu PSCs at P14-15.

4. Discussion

Epidemiological studies have stressed the importance of gestational maternal infection and inflammatory processes as risk factors for developmental disorders in offspring (Atladóttir et al., 2012; Atladóttir et al., 2010; Brown, 2012; Knuesel et al., 2014; Lyall et al., 2014; Patterson, 2009, 2002; Zerbo et al., 2016). To the best of our knowledge, our results provide the first evidence that MIA produces already at birth important physiological and morphological changes in the hippocampus of mice. These results shed new light on the deleterious effects of in-utero MIA on offspring brain physiology already at birth and its long-term consequences, and are in accordance with the recent demonstration of a delayed excitatory/inhibitory shift of GABA in P20 cortical neurons of MIA rat offspring (Corradini et al., 2017). Specifically, we show that MIA alters spontaneous activity and GABAergic polarity in hippocampal CA3 neurons from P0.

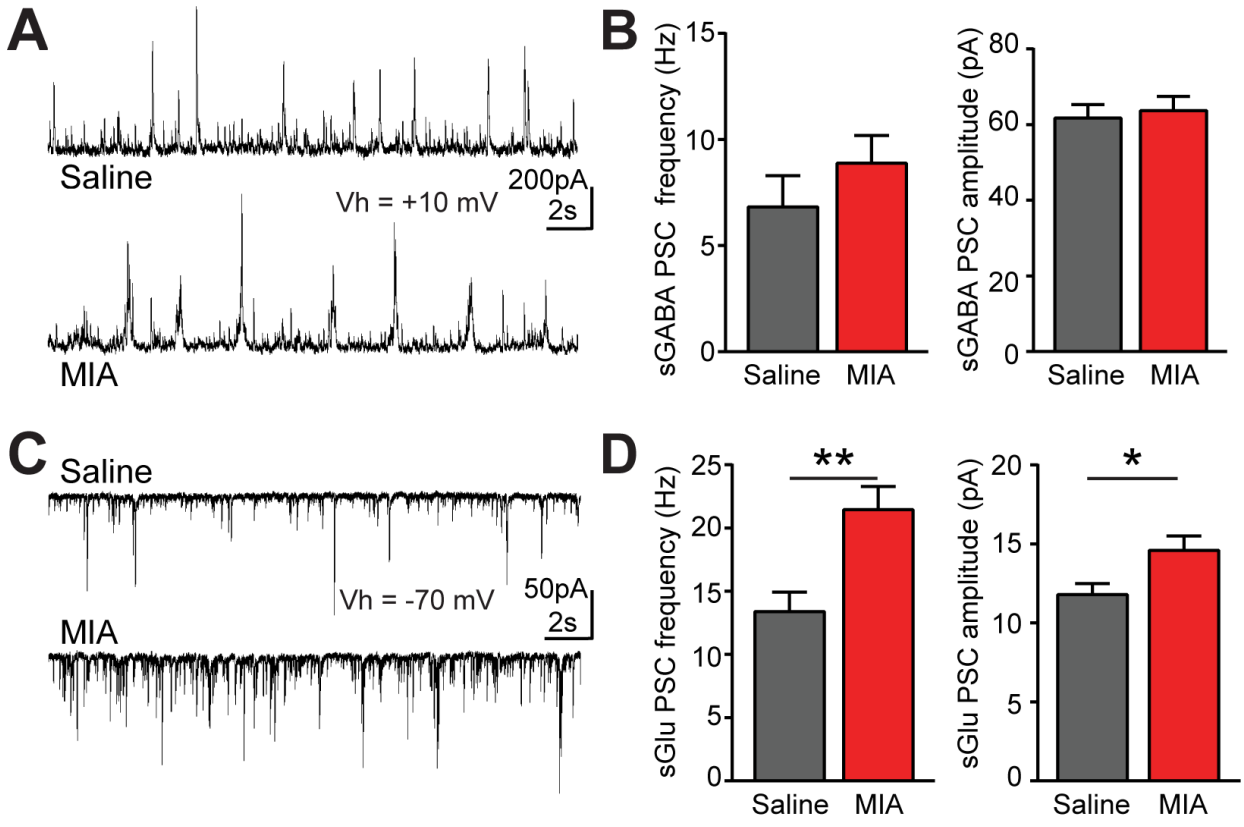

Fig. 4. Excitatory inputs are elevated at P14-15 in CA3 pyramidal neurons of MIA offspring. Representative traces of sGABA PSCs and (C) sGlu PSCs recorded in whole-cell voltage clamp configuration of P14-15 CA3 pyramidal neurons of saline or MIA offspring. (B) The frequency and amplitude of sGABA PSCs are similar between saline (n=14 cells) and MIA (n=19 cells) neurons. (D) The frequency and amplitude of sGlu PSCs are increased in MIA pyramidal neurons (n=26 cells) compared to saline (n=18 cells). Mann-Whitney Test, * $p<0.05$, ** $p<0.005$.

Birth marks the developmental transition from a stabilised environment to a continuous flow of unpredictable stimuli, therefore initiating major physiological adaptations. The foetal brain must then quickly overcome the major ongoing physiological changes, without being affected by the harmful side effects of the acute stressors during the delivery process (Lagercrantz and Bistoletti, 1977). Furthermore, dysregulated GABAergic currents on the day of birth have been identified as a critical signature of developmental brain disorders (Ben-Ari, 2015). Indeed, we previously showed that the protective inhibitory action of GABA at birth was abolished in hippocampal neurons of VPA and FRX rodent models of ASD (Tyzio et al., 2014), accompanied by lasting imbalanced sGlu PSCs and maintained depolarising and excitatory GABA. The present study reveals that MIA similarly alters GABA action at birth by dysregulating intracellular chloride concentration, as the acute application of the NKCC1 antagonist bumetanide rescued the polarity of GABA. In addition, the significant increase of ongoing GABAergic activity that we report at birth suggests that MIA leads to hyperactivity of depolarising GABAergic networks at this early post-natal period.

Our results further show that the apical morphology of hippocampal CA3 pyramidal neurons from MIA newborn offspring is hypertrophied and strongly altered in shape. Interestingly, previous investigations of neuronal morphology in MIA models showed reorganised arborisation and increased apical dendrites complexity in P14-21 mouse pyramidal cells from layer II primary visual cortex (Gao et al., 2015). By opposition, in adult MIA mouse offspring, granule cells from hippocampal dentate gyrus showed decreased total number and length of dendritic intersections (Zhang and van Praag, 2015) as well as altered dendritic architecture in medial prefrontal cortex pyramidal cells (Li et al., 2014).

In our study at birth, it is likely that sustained GABAergic activity contributes to the apical dendritic overgrowth since enhanced GABAergic activity activating $GABA_A$ and $GABA_B$ receptors was shown to modulate neuritic outgrowth in pyramidal neurons and interneurons (Barbin et al., 1993; Ben-Ari, 2002; Gaiarsa and Porcher, 2013; Owens and Kriegstein, 2002) through GABA-induced depolarisation implicating calcium-dependent mechanisms (Represa and Ben-Ari, 2005; Wang and Kriegstein, 2009). Furthermore, an abundant literature notably links GABAergic activity with the trophic factor BDNF (brain-derived neurotrophic factor) (Kuczewski et al., 2011). Hence, GABA release by hippocampal interneurons regulates BDNF release by surrounding cells, regulating in turn interneurons morphology in a paracrine manner (Marty et al., 1996). From a network perspective, increased GABA input following MIA might then favour enhanced GABAergic neurons growth and synaptogenesis as well (Fiorentino et al., 2009), therefore increasing the probability of synaptic contacts between interneurons and pyramidal cells. This could contribute to the increased probability of synchronous activity between active cellular pairs in MIA hippocampal networks, and though not significant to the increased tendency for correlated calcium plateau activities between more distant cellular pairs.

These early network alterations and the lasting imbalanced excitatory *vs.* inhibitory neurotransmission, known to be central in psychiatric disorders (Rubenstein and Merzenich, 2003; Volk and Lewis, 2013), operate by generating a vicious cycle with enhanced activity producing reactive plasticity and aberrant connectivity. In line with this is our observation of a “bi-phasic” excitatory *versus* inhibitory imbalance in MIA offspring: GABAergic but not glutamatergic currents are altered at birth, and conversely glutamatergic but not GABAergic currents are affected in pre-wean juvenile mice. Furthermore, several reports have shown alterations in the excitatory vs inhibitory balance after MIA. Indeed, adult offspring from Poly(I:C) MIA subjected to an epilepsy protocol produced by focal electrical stimulation in the hippocampus (i.e. kindling model of epilepsy) were shown to exhibit increased hippocampal excitability and accelerated kindling rate, with increased seizure susceptibility persisting two weeks later (Pineda et al., 2013). Decreased EEG coherence between dorsal hippocampus and medial prefrontal cortex was also described in hippocampal neurons from MIA adult rat offspring (Dickerson et al., 2014). Interestingly, several reports in adult MIA rodent offspring described parvalbumin GABAergic neurons impairments such as decreased GAD67 protein levels in the dorsal hippocampus (Piontkewitz et al., 2012), cell loss in the dentate gyrus (Piontkewitz et al., 2012; Zhang and van Praag, 2015) and decreased input in the medial prefrontal cortex (Canetta et al., 2016). A developmental study by Paylor and colleagues highlighted an age-dependent and region-specific reduction of perineuronal nets in the medial prelimbic cortex and amygdala of MIA offspring (Paylor et al., 2016). Furthermore, dentate gyrus granule cells from adult MIA mouse offspring showed altered intrinsic properties, as well as frequency reduction of miniature inhibitory postsynaptic currents (mIPSCs) only affecting mature (i.e. embryonically derived) granule cells (Zhang and van Praag, 2015). Thus, in-utero exposure to MIA disrupts the inhibitory/excitatory balance from birth and into adulthood.

Nevertheless, the mechanisms by which MIA initiates physiological disturbances are still poorly understood. Choi and colleagues demonstrated in a series of studies that MIA activating the interleukin-17 pathway promotes abnormal cortical development as early as E14.5 in mice (Choi et al., 2016) resulting in dysplastic cortical patches which are responsible of abnormal behaviours in adult mice (Kim et al., 2017; Shin Yim et al., 2017). Furthermore, several investigations have shown that immune activation in a non-MIA context

modulates the expression of the Na-K-Cl cotransporter NKCC1. Namely, peripheral injection of LPS in adult mice was shown to increase the expression of NKCC1 gene mRNA in murine lung and kidney (Topper et al., 1997). Similarly, exposure to interleukin-1β and TNF-α upregulates the NKCC1 gene mRNA and protein expression in human endothelial cells of umbilical vein (Topper et al., 1997) and cultured astrocytes in a dose-dependent manner (Huang et al., 2014). Since Interleukin-1β and TNF-α are two cytokines acutely increased by Poly(I:C) injection (Giovanoli et al., 2015a) an MIA-elicited inflammatory response might trigger NKCC1 overexpression, and consequently lead to the dysregulated chloride regulation at birth that we observed.

In summary, our present findings show that MIA enhances spontaneous currents and induces depolarising GABA action from birth, followed at the intermediate juvenile stage by maintained depolarising and excitatory GABA and increased excitatory inputs. These features are in accordance with previous investigations done in the VPA and FRX rodent models of autism, emphasizing possible parallelism between these distinct pathogenic situations, their deleterious sequels, and ASD outcomes.

5. Conclusions

In conclusion, CA3 hippocampal neurons of MIA offspring show already at birth changes that are reminiscent of those occurring in animal models of ASD and other disorders (Ben-Ari, 2017). Hence, despite their intrinsic different behavioural, physiological and clinical features, neurodevelopmental disorders seem to share pathophysiological commonalities, notably regarding their early manifestation and important effects on the polarity of GABAergic signals.

Competing financial interests

Y. Ben-Ari, D.C. Ferrari, N. Burnashev, R. Tyzio and N. Lozovaya are shareholders of Neurochlore, a biotech company dedicated to the development of treatments for children with autism. Y. Ben-Ari is also the CEO of the company.

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Supplementary data

Supplementary data associated with this article can be found on pages [62-64]

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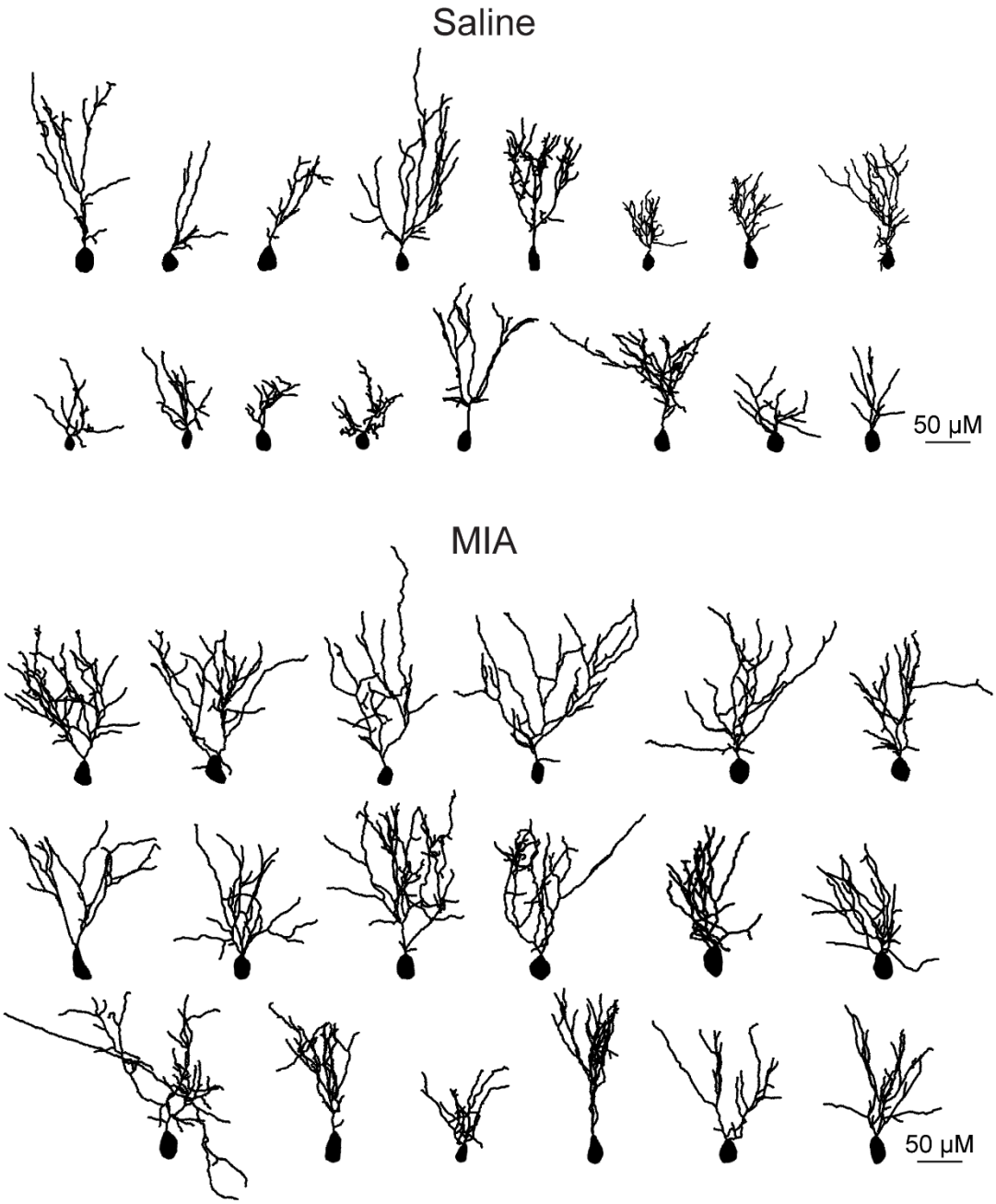
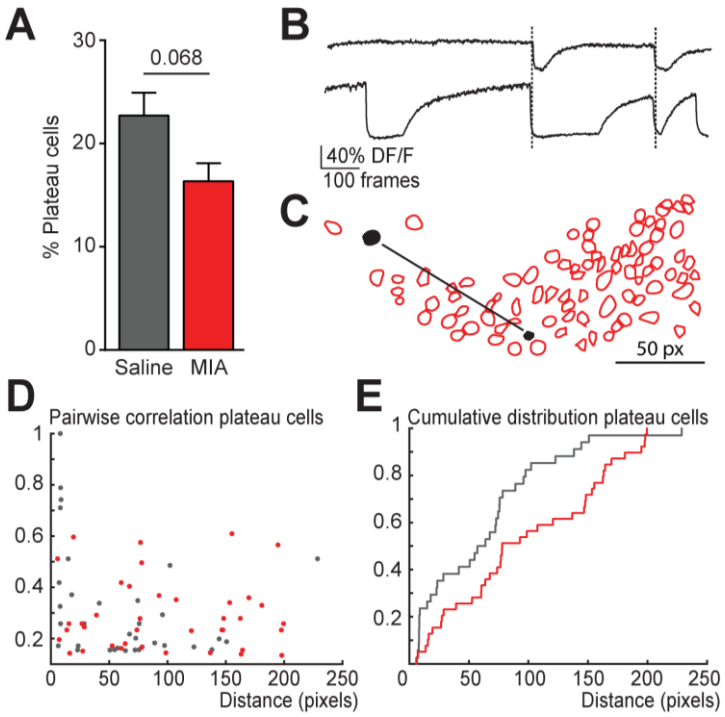
Supplementary figure 1. MIA-induced altered apical dendritic arborisation in CA3 pyramidal neurons at birth

Reconstructed apical arbours of biocytin-filled CA3 pyramidal neurons from saline (top) and MIA (bottom) P0 offspring.



Supplementary figure 2. The percentage of plateau cells and distance-dependent correlation of plateau onsets is not significantly altered by MIA at P0

(A) The percentage of cells presenting calcium plateaus is not significantly different between saline (n=15 networks) and MIA (n=14 networks) P0 CA3 networks. Mann-Whitney Test, not significant. (B) Representative traces of fluorescence emitted by correlated plateau cells loaded with Fura-2-AM. Each trace corresponds to fluorescence variations emitted by a single cell. Vertical dashed black lines mark the synchronous onset of two calcium plateaus between the two cells. (C) Representative contours of a slice loaded with Fura-2-AM with a pair of cells (black contours and filling) showing long-distance correlation between calcium plateaus events timing. (D) Scatter-plot showing the correlation between the onset of calcium events in plateau cells as function of the cell distance. Only significant correlations were plotted. (E) Cumulative distribution probability of correlated plateau cells as function of cell distance. Kolmogorov Smirnov test, not significant, p=0.06.



Supplementary table 1. Intrinsic properties of CA3 pyramidal cells at P0

Group	Saline	MIA	Saline vs MIA
Passive properties			
n	15 cells	15 cells	Mann-Whitney Test
Membrane potential (mV)	-45.68±3.61	-53.77±2.83	ns (0.0689)
Membrane resistance (GΩ)	3.41±0.67	2.63±0.27	ns (0.6827)
Capacitance (pF)	36.87±4.46	40.75±3.19	ns (0.4610)
Firing properties			
n	10 cells	10 cells	Mann-Whitney Test
AP threshold (mV)	-24.07±2.58	-27.32±1.14	ns (0.5137)
Max number AP in 1 second	1.3±0.21	1.6±0.27	ns (0.5310)
Rheobase (pA)	29.5±4.38	18.5±4.60	ns (0.1456)
Action potential properties			
n	12 cells	12 cells	Mann-Whitney Test
Half width (ms)	4.06±0.43	3.77±0.32	ns (0.6297)
Rise time (ms)	1.1±0.1	1±0.07	ns (0.6707)
Decay time (ms)	3.77±0.43	3.55±0.37	ns (0.7987)
Amplitude (mV)	32.79±3.63	41.04±2.67	ns (0.1277)

Discussion & Perspectives

Epidemiological studies have shown maternal infection, fever, or the use of some antibiotics during pregnancy to be associated with an increased risk for developmental disorders such as autism[114,187–189]and schizophrenia[120,190] in the offspring. In addition, viral and bacterial infections during pregnancy, leading to a maternal immune activation (MIA), have been shown to increase the risk for ASD when occurring preferentially at specific time-windows: a viral infection in the first trimester or a bacterial infection during the second trimester[114].

Several research teams have investigated the effects of in-utero exposure to MIA in animal models, and specifically their consequences on the brain, showing pleiotropic disturbances affecting a wide diversity of structures such as the neocortex[136,137,191,192], hippocampus[193,194], and cerebellum[152]. These observations allowed to better understand the long-term consequences of MIA in adult offspring, including heightened anxiety[123,195], impaired social-behaviour[136,137,195,196], defective pre-pulse inhibition[195], stereotyped behaviours[123,195] and impaired cognitive function[197–200] (see reference[201] for a review). In addition, altered electrophysiological properties have been described in cultured CA1 pyramidal neurons from P0-2 rats[202]. However, to the best of our knowledge, our results provide the first evidence that MIA produces already at birth important physiological and morphological changes in the hippocampus of mice, shedding light on the deleterious effects of in-utero MIA on the offspring's early brain development.

Birth, a critical period for the development of pathologies

Birth is a stressful event marking the developmental transition from a stabilised environment to a continuous flow of unpredictable stimuli. Therefore, birth initiates major physiological adaptations related to thermoregulation, air breathing, feeding and blood pressure[166,167,169]. In addition, the foetal brain must quickly overcome the major ongoing physiological changes, without being affected by the possible harmful side effects of anoxia during the passage through the birth canal[167] and by the excitatory effects of the high concentrations of catecholamines at the onset, during, and following delivery[166,203].

One way to protect the brain of these delivery-related challenges was described in rodents by our team, that showed that maternal oxytocin (OT) released at birth increases neuronal resistance to anoxic/aglycaemic episodes and induces a neuroprotective switch of GABA polarity towards hyperpolarising and inhibitory effects, through a reduction of intracellular chloride ($[Cl^-]_i$)[179]. This neuroprotective oxytocin-mediated reduction of $[Cl^-]_i$ at birth is also observed through a decrease in the threshold for pain induced by OT and bumetanide, in addition to reduced $[Cl^-]_i$ and hyperpolarising GABA in trigeminal sensory neurons of P0 rat pups[178]. Alterations in this shift of GABA polarity at birth have been shown in the VPA rat and FRX mouse models of ASD, which are accompanied by lasting imbalanced sGlu PSCs, and maintained depolarising and excitatory GABA at P15[180].

Our work presented here shows that MIA similarly alters GABA action at birth, which persists at P14-P15. This MIA-induced GABA depolarisation at birth is due to a dysregulation of the intracellular chloride concentration, since the acute application of the Na^+-K^+-Cl^- cotransporter NKCC1 antagonist bumetanide rescued the polarity of GABA.

MIA leads to altered hippocampal CA3 pyramidal cells morphology at birth

We showed that MIA pyramidal neurons present hypertrophied and altered organisation of the apical dendritic tree, and exuberant apical dendrites at birth. We also observed a significant increase of ongoing spontaneous GABAergic activity at birth. It is likely that this latter observation contributes to the apical dendritic overgrowth since enhanced GABAergic activity activating $GABA_A$ and $GABA_B$ receptors was shown to modulate neuritic outgrowth in pyramidal neurons and interneurons[81,204–208] through GABA-induced depolarisation implicating calcium-dependent mechanisms[204,209].

Reorganised arborisation of neurons and increased apical dendrites complexity following LPS-MIA have also been reported in P14-21 mouse pyramidal cells from layer II primary visual cortex[210]. In contrast, in adult Poly(I:C)-MIA mouse offspring, granule cells from hippocampal dentate gyrus showed decreased total number and length of dendritic intersections[211], as well as altered dendritic architecture in medial prefrontal cortex pyramidal cells[197]. Altogether, these morphological alterations of MIA neurons raise the hypothesis of either an MIA-induced dysregulation of neurotrophic cues, or a lack of control of the dendritic growth.

An abundant literature links GABAergic activity with brain-derived neurotrophic factor (BDNF, also referred as mature BDNF, mBDNF) and its precursor proBDNF, which belong to the protein family of mammalian neurotrophins[212].

Both forms of BDNF and their common receptors, proBDNF showing high affinity for $p75^{NTR}$ and mBDNF to tyrosine kinase receptor TrkB[213–215], are expressed in the rodent developing nervous system during times of active neuronal growth and differentiation[213,216]. Furthermore, GABA released by hippocampal interneurons has been shown to influence the release of BDNF by neighbouring cells, thus regulating in turn interneurons morphology in a paracrine manner[217].

To test the hypothesis that MIA involves BDNF signalling at birth to induce alteration of pyramidal neuron's morphology, we could check pyramidal neuron's morphology in foetal E18 hippocampi, or administer to pregnant mice the highly specific TrkB antagonist ANA-12[218,219] on the day before delivery. Additionally, BDNF protein expression, as well as its receptors, could be assessed in hippocampi of MIA and vehicle treated offspring at birth. Furthermore, since it has been shown that cultured rat microglial cells produce BDNF following stimulation with LPS[220], and Human cultured microglial cells synthesise nerve growth factor (NGF) upon stimulation with interleukin-1β and TNFα[221], we could further evaluate if MIA leads to an alteration in microglial numbers, morphology or activation at birth, and also evaluate the effects of TrkB antagonism on these parameters.

MIA alters hippocampal network activity

Early network alterations and lasting imbalanced excitatory *vs.* inhibitory neurotransmission, known to be central in psychiatric disorders[222,223], have been shown to generate a vicious cycle with enhanced activity producing reactive plasticity and aberrant connectivity[224–226]. In line with this is our observation of a "bi-phasic" excitatory *versus* inhibitory imbalance in MIA offspring: GABAergic but not glutamatergic currents are altered at birth, and conversely glutamatergic but not GABAergic currents are affected in pre-wean juvenile mice. Furthermore, several reports have shown alterations in the excitatory *vs* inhibitory balance after MIA. Indeed, adult offspring from Poly(I:C) MIA subjected to an epilepsy protocol produced by focal electrical stimulation in the hippocampus (i.e. kindling model of epilepsy) were shown to exhibit increased hippocampal excitability and accelerated kindling rate, with increased seizure

susceptibility persisting two weeks later[227]. Decreased EEG coherence between dorsal hippocampus and medial prefrontal cortex was also described in Poly(I:C) MIA adult rat offspring[228]. In addition, abnormal forebrain wiring caused by disruption of microglial control of axonal growth has been shown in embryonic mouse brains exposed to LPS-induced MIA[41].

Imbalances between excitatory/inhibitory neurotransmission can also result from the impairment of interneurons' number and function. In this work, we have mainly investigated the effects of in-utero MIA on hippocampal glutamatergic principal cells. However, several studies reported interneurons to be potently affected by MIA in rodents. Notably, PV interneurons have been shown to be particularly affected in various manners in neocortical areas, such as decreased GAD67 protein levels in the dorsal hippocampus[229], PV positive cell loss in the dentate gyrus[211,229] and decreased input in the medial prefrontal cortex[230]. The number of PV interneurons surrounded by their specific extracellular matrix, corresponding to perineuronal nets, has also been shown to present age-dependent and region-specific reductions in the medial prelimbic cortex and amygdala of MIA offspring[191]. However, principal cells of the dentate gyrus have been reported to present altered intrinsic properties, as well as frequency reduction of miniature inhibitory postsynaptic currents (mIPSCs) only affecting mature (i.e. generated embryonically, contrary to 'immature' granule cells generated during adulthood) granule cells of MIA mouse offspring[211]. Thus, in-utero exposure to MIA disrupts the inhibitory/excitatory balance from birth and into adulthood.

From a neonatal network perspective, increased GABA input following MIA might favour enhanced GABAergic neurons growth and synaptogenesis[231], therefore increasing the probability of synaptic contacts between interneurons and pyramidal cells. Consequently, this could contribute to the increased probability of synchronous activity we observed between active cellular pairs in MIA hippocampal networks and to the increased tendency ($p=0.06$) for correlated calcium plateau activities between more distant cellular pairs.

Large-scale synchronisation of network activity in the form of GDPs[100,232] is under the control of a class of specific super-connected GABAergic "hub" neurons in the rodent hippocampus during the first postnatal week[103,233]. These neurons have been shown to be early-born (specifically generated as soon as E7.5 and no later than E10.5) and their migration towards the hippocampal neuroepithelium already ongoing by E12.5 in mice[233]. Also, early-born glutamatergic neurons present as early as E12.5 have been uncovered in the CA3 area of the

mouse hippocampus and were shown to be recruited at the onset of GDPs during the first postnatal week[234]. Therefore, we can hypothesize that these early-born neurons are differentially altered by MIA compared to late-born GABAergic or principal neurons (Figure 15). To test this hypothesis, the function of hub neurons could be evaluated through the investigation of GDPs properties between P6 and P8 using the fate-mapping strategy in transgenic mice allowing the identification of early-born neurons. Indeed, Dlx1/2 and Ngn2 are transcription factors expressed transiently as the GABAergic[233,235] and glutamatergic neurons[234], respectively are becoming postmitotic. Using the tamoxifen-inducible CRE recombinase construction, neurons can be identified based on their date of birth via the expression of a reporter fluorescent protein allowing us to specifically target early-born neurons. We could expect that changes in hub neurons connectivity or synaptic efficiency could be translated into changes of GDPs frequency and number of neurons recruited, as well as morphological alterations.

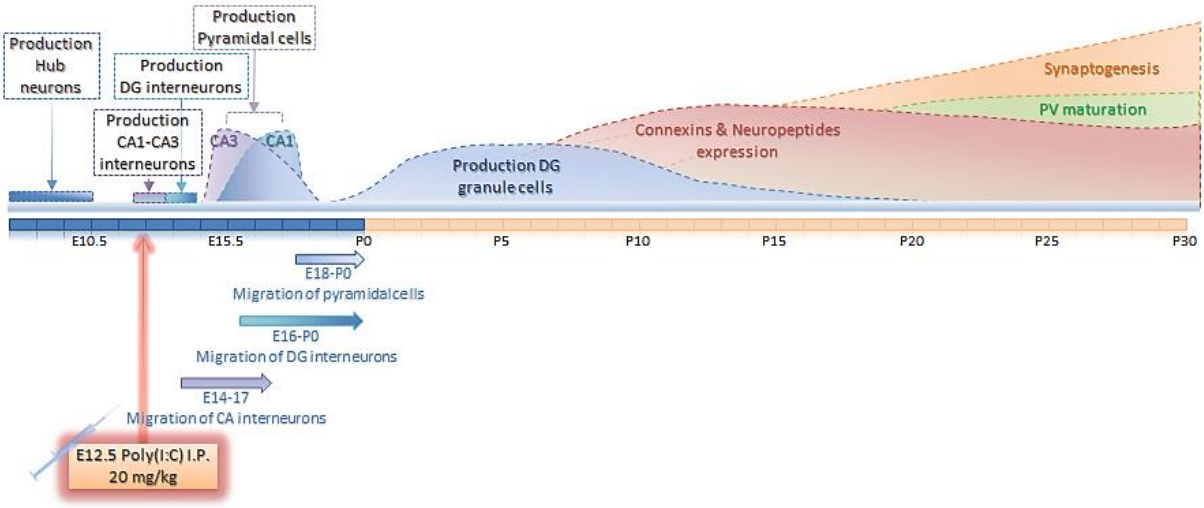


Figure 16 – Poly(I:C) injection at E12.5 corresponds with the time of interneurons generation during embryonic development and is consecutive to the time of generation of GABA hub neurons. Adapted from Danglot, L. *et al* (2006)[59].

MIA induces lasting developmental alterations

The concept of Neuro-archaeology states that neurons with immature features might constitute a leading pathogenic mechanism perturbing behaviourally relevant brain network oscillations[236]. Thus, Poly(I:C) MIA has been shown to induce the development of dysplastic

cortical patches in the embryonic brain as early as E14.5 that persist into adulthood, and whose size is correlated with the severity of MIA-induced behavioural phenotypes in adult mice[136,137]. Furthermore, we report in our study the maintenance of immature depolarising GABA action at P14-15, which corresponds to a perturbation of the GABA developmental sequence that can potentially lead to misplaced/misconnected neurons.

The regulation of $[Cl^-]_i$ by cation-chloride co-transporters (CCCs) NKCC1 and KCC2 is the pivotal mechanism on which relies the action of GABA. A growing body of evidence suggests NKCC1 deregulations to be involved in various CNS pathologies[237] accompanied by depolarising actions of GABA ranging from epilepsy[86,238] to neuropathic pain[84], including animal models of ASD[180]. Importantly, the expression of NKCC1 is modulated by various immune mediators. Indeed, peripheral injection of LPS in adult mice was shown to increase the expression of NKCC1 gene mRNA in murine lung and kidney[239]. Similarly, exposure to IL-1β and TNF-α upregulates the NKCC1 gene mRNA and protein expression in human endothelial cells of umbilical vein[239] and cultured astrocytes in a dose-dependent manner[240]. Since IL-1β and TNF-α are two cytokines acutely increased by Poly(I:C) injection[199], an MIA-elicited inflammatory response might trigger NKCC1 overexpression and consequently lead to the dysregulated chloride regulation at birth that we observed. To test this hypothesis, we could treat pregnant females with bumetanide at birth to try a rescue of the perinatal depolarising GABA. Another option would be to treat offspring postnatally from P12 onwards, corresponding to the end of the normal period for depolarising GABA[180]. We could then evaluate the effects of the treatment on the DF_{GABA} at P16, and on offspring's behaviour in adult mice.

Despite the growing data uncovering the consequences of in-utero MIA on the offspring, the mechanisms by which MIA initiates physiological disturbances are still poorly understood. Maternal immune activation triggers the release of a cocktail of immune mediators to which the foetal brain is exposed during development. The consequences on the offspring's brain and their implication in abnormal behaviour have notably been unravelled for two interleukins. Gloria Choi's team demonstrated in a series of studies that MIA activating the IL-17 pathway promotes abnormal cortical development as early as E14.5 in mice[135]. This results in dysplastic cortical patches, whose size is highly correlated to the severity of the behavioural phenotypes in adult mice[136,137]. In addition, Paul Patterson's team showed that MIA-induced IL-6 alters brain transcriptome, leading to abnormal behaviours in adult offspring[132]. However, several studies

carried out on rodent models have suggested that MIA-induced disturbances are increased when combined with embryonic, perinatal or postnatal factors. For instance, Dr. Choi's team showed that the presence of MIA-induced dysplastic cortical patches are dependent on a maternal source of IL-17 provided by a specific strain of commensal bacteria[136,137]. Furthermore, Urs Meyer's team has shown that the exposure to peripubertal stress increases MIA-induced dopaminergic dysregulations in adult offspring[241]. This study showed that both prenatal immune activation and peripubertal stress are necessary to induce sensorimotor gating deficiency and behavioural hypersensitivity to psychotogenic drugs in adult MIA mice[241]. Altogether, these reports show that MIA is a risk factor for physiological and behavioural disturbances that are aggravated when synergising factors intervene, thus constituting a double-hit insult.

Aside from the mechanism following any type of MIA, we can conclude that exposure to maternal inflammation during the embryonic development produces alterations including physiological and morphological sequelae at the critical period of birth. Notably, MIA affects the pivotal birth-associated switch of GABA polarity and produces lasting excitatory/inhibitory imbalances.

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