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# Evaluation of the effects of inflammation and combined exposure to environmental toxicants during the perinatal period : a potential etiological factor of neurodegenerative pathologies ?

Asma Oummadi

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## École doctorale Santé, Sciences Biologiques et Chimie du Vivant

Laboratoire d'Immunologie et Neurogénétique Expérimentales et Moléculaires  
UMR7355 – CNRS – Orléans

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### THÈSE EN COTUTELLE INTERNATIONALE

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pour obtenir le grade de :  
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**Evaluation of the effects of inflammation and  
combined exposure to environmental toxicants  
during the perinatal period: A potential etiological  
factor of neurodegenerative pathologies?**

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*"You took the sourest lemon life has to offer and turned it into something resembling lemonade."*

Jack Pearson – This is Us

*« La fleur qui s'épanouit dans l'adversité est la plus rare et la plus belle de toute »*

Empereur de Chine – Mulan

*« Il n'y a qu'une façon d'échouer, c'est d'abandonner avant d'avoir réussi »*

Georges Clémenceau

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# PUBLICATIONS

2023

Asma Oummadi, Flora Reverchon, Arnaud Menuet, Anthony Delalande, Séverine Morisset-Lopez, Paola Bezzi, Gilles J. Guillemain, Stéphane Mortaud and Anthony Laugeray\*. Refinement of behavioural experiments through 3R-based and ethologically relevant paradigms in mice. *Hypothesis and Theory, Front. Behav. Neurosci. - Emotion Regulation and Processing*. Manuscript ID: 1219889 (submitted)

Asma Oummadi\*, Arnaud Menuet, Sarah Méresse, Anthony Laugeray, Stéphane Mortaud. Maternal exposure to a pollutant mixture induces long lasting motor coordination impairment and emotional disturbances in offspring's: importance of considering "cocktail effect". (In preparation)

Asma Oummadi\*, Arnaud Menuet, Sarah Méresse, Anthony Laugeray, Gilles J. Guillemain and Stéphane Mortaud. The herbicides Glyphosate and Glufosinate and the cyanotoxin BMAA induce long-term motor disorders following postnatal exposure: importance of a prior asymptomatic maternal inflammatory sensitization. *Front. Neuroscience. – Neurodevelopment (2023)* Manuscript ID: 1172693 DOI=10.3389/fnins.2023.1172693

Jobin ML, De Smedt-Peyrusse V, Ducrocq F, Baccouch R, Oummadi A, Pedersen MH, Medel-Lacruz B, Angelo MF, Villette S, Van Delft P, Fouillen L, Mongrand S, Selent J, Tolentino-Cortez T, Barreda-Gómez G, Grégoire S, Masson E, Durroux T, Javitch JA, Guixà-González R, Alves ID, Trifilieff P. Impact of membrane lipid polyunsaturation on dopamine D2 receptor ligand binding and signaling. *Mol Psychiatry*. 2023 Jan 6. doi: 10.1038/s41380-022-01928-6. Epub ahead of print. PMID: 36604603.

2022

Méresse S, Larrigaldie V, Oummadi A, de Concini V, Morisset-Lopez S, Reverchon F, Menuet A, Montécot-Dubourg C, Mortaud S.  $\beta$ -N-Methyl-Amino-L-Alanine cyanotoxin promotes modification of undifferentiated cells population and disrupts the inflammatory status in primary cultures of neural stem cells. *Toxicology* 482:153358 (2022) doi: 10.1016/j.tox.2022.153358 hal-03841716v1

## 2020

**Oummadi A**, Meyer-Dilhet G, Béry A, Aubert A, Barone P, Mortaud S, Guillemin GJ, Menuet A, Laugeray A. 3Rs-based optimization of mice behavioral testing: The habituation/dishabituation olfactory test. *J Neurosci Methods*. 2020 Feb 15;332:108550. doi: 10.1016/j.jneumeth.2019.108550. Epub 2019 Dec 12. PMID: 3183818

Ducrocq F, Walle R, Contini A, **Oummadi A**, Caraballo B, van der Veldt S, Boyer ML, Aby F, Tolentino-Cortez T, Helbling JC, Martine L, Grégoire S, Cabaret S, Vancassel S, Layé S, Kang JX, Fioramonti X, Berdeaux O, Barreda-Gómez G, Masson E, Ferreira G, Ma DWL, Bosch-Bouju C, De Smedt-Peyrusse V, Trifilieff P. Causal Link between n-3 Polyunsaturated Fatty Acid Deficiency and Motivation Deficits. *Cell Metab*. 2020 Apr 7;31(4):755-772.e7. doi: 10.1016/j.cmet.2020.02.012. Epub 2020 Mar 5. PMID: 32142670.

## 2019

Ducrocq F, Hyde A, Fanet H, **Oummadi A**, Walle R, De Smedt-Peyrusse V, Layé S, Ferreira G, Trifilieff P, Vancassel S. Decrease in Operant Responding Under Obesogenic Diet Exposure is not Related to Deficits in Incentive or Hedonic Processes. *Obesity (Silver Spring)*. 2019 Feb;27(2):255-263. doi: 10.1002/oby.22358. Epub 2018 Dec 30. PMID: 30597761.

## 2018

Laugeray A, **Oummadi A**, Jourdain C, Feat J, Meyer-Dilhet G, Menuet A, Plé K, Gay M, Routier S, Mortaud S, Guillemin GJ. Perinatal Exposure to the Cyanotoxin  $\beta$ -N-Méthylamino-L-Alanine (BMAA) Results in Long-Lasting Behavioral Changes in Offspring-Potential Involvement of DNA Damage and Oxidative Stress. *Neurotox Res*. 33:87-112 (2018). doi: 10.1007/s12640-017-9802-1. PMID: 28879461. (co-first author with Laugeray A)

# SCIENTIFIC COMMUNICATIONS

## *2022 International:*

Oummadi A, Laugeray A, Guillemin G, Mortaud S. Interaction between a mild inflammatory episode and a perinatal exposure to a pollutant mixture induces long lasting motor coordination impairment and emotional disturbances in offsprings. FENS Forum International Neuroscience Conference 2022, 9-13 July 2022, Paris, FR. (**poster**)

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Oummadi A, Laugeray A, Guillemin G, Mortaud S. “Interaction between a mild inflammatory episode and a perinatal exposure to a pollutant mixture induces long lasting motor coordination impairment and emotional disturbances in offsprings”. Neurodevelopmental Disorders: From molecular mechanisms to social inclusion, 7TH BORDEAUX NEUROCAMBUS CONFERENCE- 18-20 MAY 2022, Bordeaux, FR (**Poster**)

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Oummadi A, Laugeray A, Guillemin G, Mortaud S. Interaction between a mild inflammatory episode and a perinatal exposure to a pollutant mixture induces long lasting motor coordination impairment and emotional disturbances in offspring. ANS Conference 2022, 6-8 December 2022, Paris, FR. (**poster – remotely**)

## *2020 National:*

Oummadi A. "Evaluation of the effects of inflammation and combined exposure to environmental toxicants during the perinatal period: A potential etiological factor of neurodegenerative pathologies? » SFR Neurosciences, 16 Nov 2020, Tours (**oral presentation, remotely**)

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# LIST OF ABBREVIATION

Abbreviation	Definition
AD	Alzheimer Disease
ADHD	Attention-deficit/hyperactivity disorder
AEG	N-(2-aminoethyl)glycine
ALS	Amyotrophic Lateral Sclerosis
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
ASD	Autism Spectrum Disorder
AUC	Air Under the Curve
BBB	Blood Brain Barrier
BGS	Brain Growth Spurt
BMAA	$\beta$ -N-Methylamino-L-Alanine
CNS	Central Nervous System
CTL	Control
DA	Dopamine
DAB	2,4-diaminobutyric acid
DAP	2,3-diaminopropionic acid
DNA	Deoxyribonucleic acid
DOHaD	Developmental Origin of Health and Disease
DOPD	Developmental Origins of Parkinson's Disease
DSM-5	Manual of Mental Disorders, 5th Edition
ECHA	European Chemicals Authority
ED	Embryonic Day
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EPSC	Excitatory postsynaptic potentials
EPSPS	Enzyme5-enolpyruvoyl-shikimate-3-phosphate synthase
FAO	Food and Agriculture Organization
GABA	Gamma-aminobutyric
GD	Gestational Day
GLA	Glufosinate Ammonium
GLUR 1/5	Glutamatergic receptor 1/5
GLY	Glyphosate
HRAC	Herbicide Resistance Action Committee
IARC	International Agency for Research on Cancer
IL-1 $\beta$	Interleukin 1 $\beta$
IL-6	Interleukin 6
IN	Intranasal
INERIS	Institut national de l'environnement industriel et des risques

IP	Intraperitoneal
KP	Kynurenine Pathway
LOAEL	Lowest Observable Adverse Effect Level
LPS	Lipopolysaccharide
MAA	Marketing Authorization Application
MCPG	Metabotropic glutamate receptor antagonist
MIA	Maternal Immune Activation
N2	Di Azote
NCP	Neural Progenitor Cells
NDD	Neurodevelopmental Disease
NE	Neuro Epithelial
NEC	Neuroepithelial cells
NMDA	N-méthyl-D-aspartate
NO	Nitric Oxide
NOAEL	No Observable Adverse Effect Level
OB	Olfactory Bulb
PD	Parkinson Disease
PDC	Parkinson Dementia Complex
PND	Post Natal Day
PPT	Phosphynotricin
RGC	Radial glial cells
ROS	Reactive oxygen species
SN	Substantia Nigra
SVZ	Sub Ventricular Zone
TDP-43	TAR DNA-binding protein 43
VDCC	Voltage-dependent calcium channels



# Résumé de la thèse en Français

## PRÉFACE

Cette thèse a été réalisée au sein de l'INEM, une unité de recherche ayant deux grands thèmes liés à la neuroscience et à l'immunologie, au sein de l'équipe "Neurotoxicité et développement". Les études ont également été menées au sein du Centre de Recherche sur les Maladie Dégénératives des neurones moteurs (MND Research center), au sein du groupe de Neuro-inflammation, à l'Université Macquarie de Sydney. Historiquement, ces deux équipes se sont intéressées à l'implication des polluants environnementaux dans les processus neuro-inflammatoires ainsi que dans l'émergence de perturbations neuronales. Ainsi, la collaboration est née d'un intérêt commun pour deux polluants spécifiques présentant des effets neurotoxiques : une cyanotoxine, la bêta-Méthylamino-L-alanine (BMAA) et un herbicide, le Glufosinate Ammonium. À travers cet intérêt commun, l'objectif était de fournir des réponses complémentaires concernant l'implication de l'exposition à ces molécules dans l'émergence de troubles du comportement, notamment dans le contexte de la discussion actuelle sur l'exposome.

Dans ce manuscrit, l'ensemble de mon travail est synthétisé en trois parties principales. Tout d'abord, en introduction, j'ai choisi d'aborder les étapes majeures du neurodéveloppement, y compris l'établissement du système nerveux central. Cela m'a permis de souligner l'importance des périodes prénatale et postnatale, dont la perturbation est particulièrement impliquée dans le développement de divers troubles neurologiques. Ensuite, les pathologies neuro-développementales, ainsi que certaines maladies neurodégénératives telles que la sclérose latérale amyotrophique ou la maladie d'Alzheimer, ont été discutées en termes de leurs signes cliniques et étiologiques, en se concentrant d'abord sur les facteurs génétiques puis en explorant les influences environnementales. Cela me permet, dans la partie suivante de cette introduction, de développer les facteurs environnementaux impliqués dans ces pathologies neurologiques. Après avoir discuté des généralités concernant les pesticides et les cyanobactéries, je décris leur implication dans le développement de troubles neurologiques. Enfin, je me tourne vers le concept d'exposome, en considérant l'exposition combinée à ces facteurs plutôt qu'en les examinant séparément.

Dans un second temps, la deuxième partie majeure concerne la présentation des principaux résultats obtenus au cours de cette thèse. La première partie décrit l'importance de la sensibilisation inflammatoire maternelle dans l'apparition de troubles moteurs chez la descendance suite à une exposition postnatale à des xénobiotiques (Asma Oummadi et al., 2023). La deuxième partie explore l'importance de prendre en compte l'effet cocktail dans l'émergence de troubles du comportement chez la descendance suite à l'exposition maternelle à un mélange de polluants environnementaux (Asma Oummadi et al., en cours de préparation).

Enfin, ce manuscrit de thèse se conclut par une discussion générale sur les succès et les limites de ce travail. Ainsi, je reviens sur les principaux résultats obtenus, mettant en évidence non seulement l'importance du concept de multi-exposition, mais aussi la vulnérabilité de la période prénatale. Ces insights soulèvent de nouvelles questions et présentent des perspectives de recherche originales.

# HYPOTHESE DE TRAVAIL ET PRINCIPAUX OBJECTIFS DE THESE

Au cours des dernières décennies, il a été observé un intérêt croissant pour l'implication des facteurs environnementaux dans l'éthologie des pathologies neuro-développementales et/ou neurodégénératives. Bien que la recherche ciblant la période périnatale soit courante dans la littérature, notamment en ce qui concerne les effets de l'inflammation maternelle à l'aide du modèle d'Activation Immunitaire Maternelle (AIM), peu intègrent la notion de faibles doses. En effet, les effets observés sont souvent liés à l'utilisation de doses particulièrement élevées, ne représentant pas nécessairement celles auxquelles nous sommes exposés quotidiennement. Les rares études utilisant des doses relativement faibles ne montrent pas d'effets clairs et durables chez les descendants. De plus, la recherche sur ces pathologies s'est principalement concentrée sur l'exposition à un seul facteur (pesticides, cyanotoxines ou inflammation maternelle), en négligeant la notion d'exposome et d'expositions multiples, qui revêtent une importance capitale dans l'émergence de ces troubles.

Cette thèse vise à aborder les conditions d'exposition aussi près que possible de la réalité. Par conséquent, comme cela n'a jamais été évalué à notre connaissance, nous avons émis l'hypothèse qu'une exposition combinée au glyphosate, au glufosinate à d'ammonium et à la BMAA, associée ou non à une sensibilisation inflammatoire, pourrait agir en synergie et induire des effets neurobiologiques délétères. Ainsi, afin de mieux répondre à cette hypothèse, les principaux objectifs de cette thèse étaient, tout d'abord, de déterminer le lien entre l'exposition à des agents environnementaux et le développement tardif de troubles neurologiques. Parallèlement, l'autre objectif principal était de développer un protocole expérimental, se rapprochant des "conditions réelles d'exposition", en plaçant notre étude dans un contexte d'exposome. Cela consistait en une exposition chronique et à faible dose concomitante à différents facteurs environnementaux, associée ou non à un épisode inflammatoire léger.

Afin d'évaluer cet effet synergique, divers protocoles *in vivo* ont été mis en place. Dans ce contexte, le premier protocole *in vivo* consistait à savoir si un épisode inflammatoire

sensibiliserait une exposition post-natale à de faibles doses de substances toxiques, afin d'explorer l'hypothèse du « multiple-hits ». Le deuxième, axé sur l'évaluation de l'effet "cocktail", consistait en une exposition maternelle à de faibles doses de BMAA, de GLY ou de GLA, soit séparément, soit en mélange.

Par la suite, une évaluation comportementale longitudinale a été réalisée chez les descendants à différents moments, de la période post-natale au vieillissement, sur différentes sphères comportementales, y compris le comportement moteur. L'ensemble de ce travail offre une perspective innovante concernant l'implication des expositions multiples dans l'évaluation toxicologique des effets à long terme.

# RESUME DE LA PREMIERE ETUDE

**Article 1** : Les herbicides Glyphosate et Glufosinate ainsi que la cyanotoxine BMAA induisent des troubles moteurs à long terme suite à une exposition postnatale : importance d'une préalable sensibilisation inflammatoire maternelle asymptomatique (accepté pour publication)

**Contexte** : L'Activation Immunitaire Maternelle (AIM) prénatale et/ou l'exposition périnatale à divers xénobiotiques ont été identifiées comme des facteurs de risque de troubles neurologiques, notamment de maladies neurodégénératives. Les données épidémiologiques suggèrent une association entre de multiples expositions précoces à diverses agressions et les neuropathologies. L'hypothèse des "multiple-hits" suppose que l'inflammation prénatale rend le cerveau plus susceptible à une exposition ultérieure à plusieurs types de neurotoxines. Pour explorer cette hypothèse et ses conséquences pathologiques, une procédure comportementale longitudinale a été réalisée après une sensibilisation prénatale et une exposition postnatale à de faibles doses de polluants.

**Méthodes** : Une exposition maternelle à un challenge immunitaire aigu (premier hit) a été induite par une dose asymptomatique de LPS (0,008 mg/kg) chez les souris. Cette sensibilisation a été suivie d'une exposition postnatale des descendants à des produits chimiques environnementaux (deuxième hit) par voie orale. Les produits chimiques utilisés étaient de faibles doses de la cyanotoxine  $\beta$ -N-Méthylamino-L-Alanine (BMAA ; 50 mg/kg), de l'herbicide Glufosinate Ammonium (GLA ; 0,2 mg/kg) ou du pesticide Glyphosate (GLY ; 5 mg/kg). Après l'évaluation des paramètres maternels, une évaluation comportementale longitudinale a été réalisée sur la descendance afin d'évaluer les capacités motrices et émotionnelles à l'adolescence et à l'âge adulte.

**Résultats** : Tout d'abord, nous avons montré que la faible stimulation immunitaire au LPS était une asymptomatique. Bien qu'une augmentation significative des cytokines pro-inflammatoires systémiques ait été détectée chez les mères, aucun défaut comportemental maternel n'a été observé. De plus, comme le montrent les tests de rotarod et le dispositif Openfield, cette administration prénatale de LPS seule n'a pas montré de perturbation

comportementale chez la descendance. Fait intéressant, nos données ont montré que la descendance soumise à la fois à l'AIM et à une exposition postnatale à la BMAA ou au GLA présentait des altérations du comportement moteur et de l'anxiété à l'adolescence et à l'âge adulte. Cependant, cet effet synergique n'a pas été observé chez la descendance exposée au GLY.

**Conclusion :** Ces données ont démontré que la sensibilisation immunitaire prénatale et asymptomatique devrait être un effet d'amorçage pour une exposition ultérieure à de faibles doses de polluants. Ces doubles coups agissent en synergie pour induire des phénotypes liés aux maladies des motoneurones chez la descendance. Ainsi, nos données mettent fortement en lumière la nécessité de prendre en compte les expositions multiples dans l'évaluation réglementaire de la neurotoxicité du développement et ouvrent la voie à des études futures visant à décrypter les voies cellulaires impliquées dans ces processus de sensibilisation.

# RESUME DE LA DEUXIEME ETUDE

Article 2 : L'exposition maternelle à un mélange de polluants induit des altérations durables de la coordination motrice et des perturbations émotionnelles chez la descendance : importance de prendre en compte l'effet "cocktail" (en cours de préparation)

**Contexte :** Tout au long de sa vie, un individu est exposé à une multitude de facteurs sociaux et environnementaux qui définissent leur exposome. Un nombre croissant d'études tentent d'incorporer ce concept dans la compréhension des troubles neurologiques. Les données épidémiologiques suggèrent une association entre de multiples expositions précoces à diverses agressions et les neuropathologies. L'effet "cocktail" suppose que l'exposition simultanée à plusieurs produits chimiques, même à faible dose, peut avoir des effets encore plus néfastes sur la santé humaine. Pour explorer cette hypothèse et ses conséquences pathologiques, une procédure comportementale longitudinale a été réalisée après une exposition maternelle à de faibles doses de polluants, seuls ou sous forme de cocktail par voie intranasale.

**Méthodes :** Une exposition maternelle chronique à des produits chimiques environnementaux a été réalisée par voie intranasale du quatrième jour de gestation à la naissance. Les produits chimiques utilisés étaient de faibles doses de la cyanotoxine  $\beta$ -N-Méthylamino-L-Alanine (BMAA ; 50 mg/kg), de l'herbicide Glufosinate Ammonium (GLA ; 0,2 mg/kg) et du pesticide Glyphosate (GLY ; 5 mg/kg), en cocktail ou séparément. Après l'évaluation des paramètres maternels, une évaluation comportementale longitudinale a été réalisée chez la descendance afin d'évaluer les capacités motrices et émotionnelles au stade post-natal, à l'adolescence et à l'âge adulte.

# CONCLUSION

L'objectif de ma thèse était d'établir un modèle multifactoriel prenant en compte plusieurs dimensions, notamment les expositions multiples et précoces pendant la période périnatale à faibles doses. De plus, la notion de sensibilisation inflammatoire a joué un rôle important dans ce projet, mettant en évidence le concept de "multiple-hits". Une autre caractéristique majeure était l'évaluation du comportement avec un suivi longitudinal. En effet, les effets d'une telle exposition ont été évalués pendant les périodes précoces (postnatales), l'adolescence, l'âge adulte et plus tard lors du vieillissement, lorsque cela était possible.

Pour étudier tous ces aspects, deux études complémentaires ont été menées. La première approche a exploré le concept de sensibilisation inflammatoire pour déterminer si un épisode inflammatoire asymptomatique pendant la période gestationnelle sensibilisait l'individu à une exposition ultérieure à de faibles doses de xénobiotiques. La deuxième approche a évalué l'"effet cocktail" en exposant des femelles enceintes pendant la période prénatale soit à un cocktail de substances toxiques environnementales, soit individuellement, soit sous forme de mélange.

En ce qui concerne la première étude, nous avons démontré qu'une sensibilisation immunitaire prénatale et asymptomatique devrait être un effet d'amorçage pour une exposition ultérieure à de faibles doses de polluants. Ces doubles coups agissent en synergie pour induire des phénotypes liés aux maladies des motoneurones chez la descendance. Dans la continuité de notre travail, nous avons montré qu'une exposition maternelle à de faibles doses de xénobiotiques sous forme de cocktail induit des effets synergiques, précoces et durables de ces composés sur les performances motrices chez la descendance, ce qui n'est pas le cas avec les composés individuels.

Ces deux études démontrent donc que non seulement l'effet de la multi-exposition est essentiel à prendre en compte, mais aussi que la période d'exposition l'est, et en particulier la période gestationnelle, particulièrement sensible à l'exposition à de telles combinaisons de substances toxiques. Ces résultats soulèvent un important enjeu de santé publique en termes d'évaluation des effets toxicologiques de diverses molécules, nécessitant des investigations cliniques, précliniques et épidémiologiques ultérieures tenant compte des expositions



multiples à plusieurs agressions. Par conséquent, cela souligne fortement la nécessité de prendre en compte les expositions multiples pour la neurotoxicité du développement, faisant référence à l'"exposome maternel", et ouvre la voie à des études futures visant à décrypter les voies cellulaires impliquées dans ces phénomènes de sensibilisation, conduisant finalement à la construction de nouvelles voies de résultats indésirables.

Malgré les limitations rencontrées dans la conception du paradigme de l'exposome, les modèles animaux restent essentiels pour étudier l'impact des expositions environnementales sur la santé. Ils complètent souvent les approches in vitro et épidémiologiques, contribuant à une compréhension globale de l'exposome et de ses effets.

# PREFACE

This thesis was carried out within the INEM, a research unit with two major themes related to neuroscience and immunology, in the “Neurotoxicity and development” team. The studies were also conducted within the MND Research Center, in the Neuroinflammation group, at Macquarie University in Sydney. Historically, these two teams have been interested in the involvement of environmental pollutants in neuroinflammatory processes as well as in the emergence of neuronal disturbances. Thus, the collaboration arose from a shared interest in two specific pollutants that exhibit neurotoxic effects : a cyanotoxin, the beta-Methylamino-L-alanine (BMAA) and an herbicide, the Glufosinate Ammonium. Through this common interest, the objective was to provide complementary answers regarding the implication of exposure to these molecules in the emergence of behavioral disorders, particularly in the context of the current discussion surrounding the exposome.

In this manuscript, my entire work is synthesized into three main parts. Firstly, as an introduction, I chose to first address the major steps of neurodevelopment, including the establishment of the central nervous system. This allowed me to emphasize the importance of the pre- and post-natal periods, whose disruption is particularly involved in the development of various neurodisorders. Subsequently, neurodevelopmental pathologies, as well as certain neurodegenerative diseases such as Amyotrophic Lateral Sclerosis or Alzheimer's disease, were discussed in terms of their clinical signs and etiologies, initially focusing on genetic factors and then exploring environmental influences. This allows me, in the subsequent part of this introduction to develop the environmental factors involved in these neurological pathologies. After discussing generalities regarding pesticides and cyanobacteria, I describe their involvement in the development of neurological disturbances. Finally, I focus towards the concept of exposome, considering the combined exposure to these factors rather than examining them separately.

Secondly, the second major part concerns the presentation of the main results obtained during this thesis. The first part describes the importance of maternal inflammatory sensitization in the appearance of motor disturbances in offspring following postnatal exposure to xenobiotics ([Asma Oummadi et al., 2023](#)). The second part explores the importance of

considering the cocktail effect in the emergence of behavioral disturbances in offspring following maternal exposure to a mixture of environmental pollutants (Asma Oummadi et al., in preparation).

Finally, this thesis manuscript concludes with a general discussion on the successes and limitations of this work. Thus, I returned to the main results obtained, highlighting not only the importance of multi-exposure concept but also the vulnerability of the prenatal period. These insights raise new inquiries and present original research perspectives.

# Part 1: INTRODUCTION

## Chapter 1: Pre and post-natal periods: the developmental brain

### I. Establishment of the vertebrate nervous system

#### 1. Embryonic and post-natal brain development

##### A. Neurogenesis: general definition

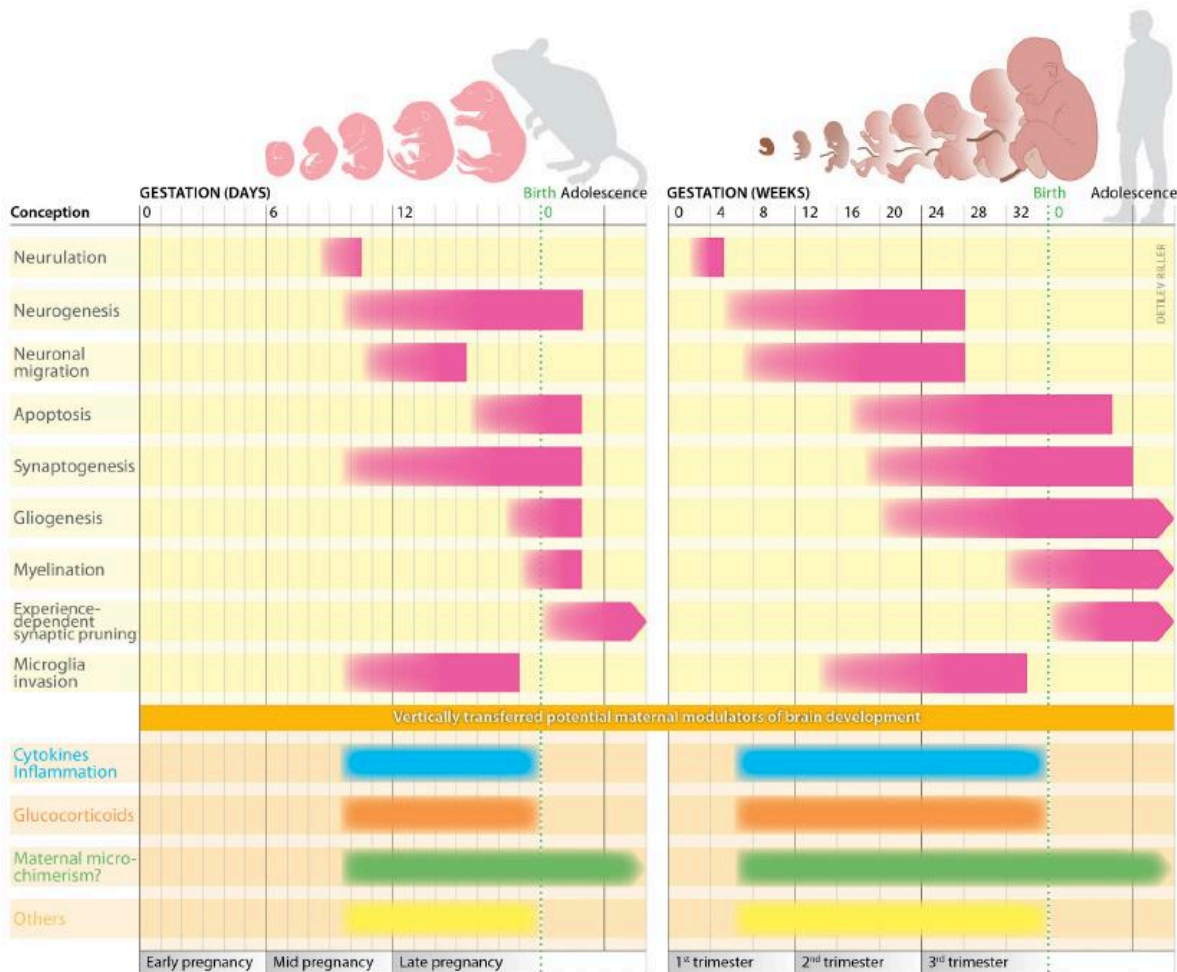
Neurogenesis is a well described and preserved mechanism preserved found in both invertebrates and vertebrates. Neurogenesis describes all the processes of cell proliferation, migration and differentiation that lead to the effective establishment of neural networks. Neurogenesis results from the activity of Neural Stem Cells (NSCs)/Neural Progenitor Cells (NCPs) which are multipotent cells capable of self-renewing and generating other cell types of the nervous system. NSC/NCP proliferation allows direct and/or indirect generation of neurons and glial cells (astrocytes and oligodendrocytes). NSC/NCP are therefore cells that can generate neurons and glial cells. In mammalian development, there are two broad categories of neural stem cells: (1) neuroepithelial cells (NE) which will rapidly yield and (2) radial glial cells (RGC). Most neurogenesis occurs during embryogenesis. However, since the beginning of the 21<sup>st</sup> century and thanks to advances in research techniques, many scientists have demonstrated a continuous neurogenesis throughout the life of the individual and have thus challenged a well-established dogma in neuroscience.

##### a. Embryonic neurogenesis

During embryogenesis, the neurulation phase consists on the establishment of the nervous system. Neurulation corresponds to the process by which a thickening of the ectoderm in the dorsal part of the embryo delimits the future neural plate, from which the whole nervous system derived, and under the influence of morphogenetic movements closes on it-even to create the neural tube. The neural tube is at the origin of the Central Nervous System (CNS) whose the anterior part will lead to the formation of five cephalic vesicles:

- The telencephalon (cortex, hippocampus, OlfactoryBulbs (OB))
- The diencephalic (optical vesicles, thalamus, hypothalamus)
- The mesencephalon (quadri-twin tubercles, cerebral peduncle)
- The metencephalon (cerebellum, Varole bridge)

- The myelencephal (spinal bulb).



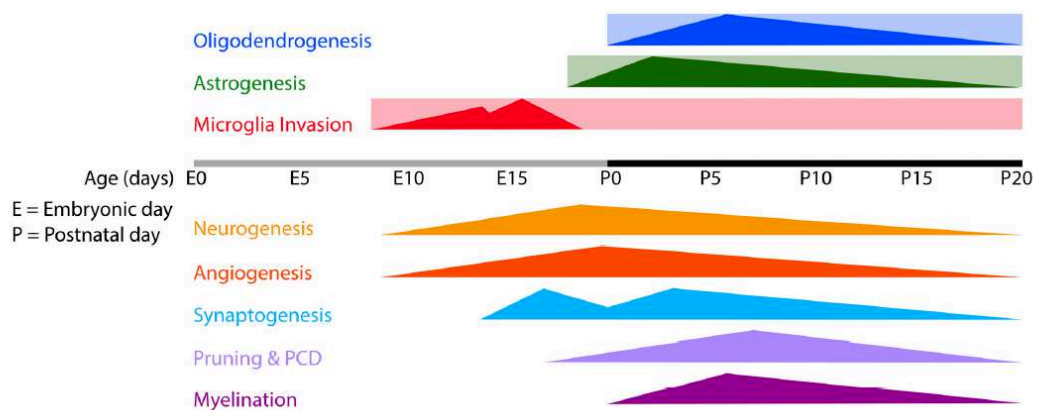
**Figure 1** – Milestone of brain development in mice (left) and humans (right). In both species, brain development starts with the formation of the neural tube (neurulation). (Schepanski et al., 2018)

Neurulation is complete around 28 days in the human embryo and around 9 embryonic days (ED) in the rodent model (**Figure 1**). Neurogenesis begins at the end of neurulation when stem cells in the neural tube proliferate massively and differentiate to form all glial cells and neurons in the nervous system. The neural tube cavity, formed during the neurulation process, constitutes the future brain's ventricles and the spinal cord ependymal canal. It is within this cavity the cerebrospinal fluid will flow. This cavity constitutes the internal base of the nervous system. Initially, a single cell layer, called the neuroepithelium, forms the nervous tissue. This neuroepithelium is composed by neuroepithelial cells (NECs) that will proliferate on the edges of the ventricles and extend themselves outwards. Cell divisions occur near the neural tube light and future neurons then migrate from the center to the periphery of the tube. These proliferative cells from neuroepithelium are by definition NSCs/NCPs.

In humans, embryonic neurogenesis occurs between the 10th and 20th week of pregnancy. Within *in vivo* model, like the mouse, this phenomenon begins around ED 10 and finishes around ED 17.

## B. Gliogenesis

Gliogenesis is described as the generation of non-neuronal glial cells resulting from Neural NCSs. Glial cells include astrocytes, oligodendrocytes, ependymocytes and microglia. Within the nervous system, glial cells (from the Greek γλοιός (gloios), «slime») constitute the neuronal environment and are fundamental in the establishment and control of synaptic activities. They are responsible for maintaining homeostasis, producing myelin, and playing a key role in supporting and protecting nervous tissue. They provide nutrients and oxygen to neurons, eliminate dead cells, and combat pathogens, ensuring the overall health and functionality of the nervous system.



**Figure 2** – Timeline of pre and post-natal cellular developmental processes in the developing mouse brain. Rectangles indicate the estimated periods during which glial cells remain present in the brain. Triangles indicate the onset and peaks of different developmental processes. (Reemst et al., 2016)

In rodents, gliogenesis begins during the embryonic period after the primary neurogenesis and persists even when it ceased (**Figure 2**). Gliogenesis and neurogenesis have in common neuro-epithelial cells of the neural tube, located in the ventricular zone.

### *a. Microglia: the first glial cells of the brain*

Microglia are the first glial cells invaded the embryonic brain, developing along with neurons during the early embryonic brain development. A theory first proposed by Alliot et al, in 1999 (Alliot et al., 1999), and then confirmed by several teams in the early 2010's (Kierdorf et al., 2013) states that microglia cells derived from yolk-sac primitive myeloid progenitor cells. Microglial colonization and migration into CNS during mouse embryogenesis is believed to occur in two phases:

- The first wave take place between ED8.5 and ED14.5 during which microglia progenitors start to invade brain tissue where it can be observed a steady increase of microglial cells. This gradual increase in microglia cells is likely induced by a rapid proliferation together with new microglia precursor's invasion.
- Between ED14 and ED16, the second wave consists in a massive and rapid increase of the number of microglial cells that could be explain by the invasion of new progenitors from the periphery. This increase seems to slow down around ED17.5.

During embryogenesis, microglia proliferate and then in constantly renewing itself throughout life to maintain their cell numbers without any contributions from bone marrow derived macrophages. Microglia development starts relatively early within embryonic life (between ED 10- ED12). Different stages have been identified depending by different sets of genes expression at the time. During the course of embryonic brain development, microglial cells disperse from the neuroepithelium to be re-distributed mainly in four specific "hotspots" where they play different key-roles:

- In the Sub-Ventricular Zone (SVZ) to control and to regulate precursor cell size
- Around blood vessels to contribute to angiogenesis
- In the choroid plexus to be involved in the phagocytic activity
- Near to developing axons to control their development and guidance

### *b. Astrocytes: the second glial cells present in the brain*

Astrocytes, like neurons, originate from neural stem cells (NSCs) in the sub-ventricular zone (SVZ). Through the final stages of neurogenesis, pluripotent radial glia can lead to astrocyte formation, which can then enter in division and proliferation phase. Astrocyte production begins around GD18 in rodents and keep going to about PND7 (**Figure 2**). Thus, during the perinatal period the astrocytes provide a physical scaffold for neuronal migration, promote the synapse formation and regulate neuronal differentiation/proliferation. Astrocytes representing the most abundant cell type in the adult brain play a fundamental role in the functioning and survival of neurons by providing a wide range of essential functions. These cells play a key-role in maintaining the blood-brain barrier, ensuring nutrients supply and the energetic metabolism of the nervous system. Astrocytes participate also in neurotransmission, detoxification of the extracellular medium, in particular by glutamate capture, and maintenance of the ionic balance of the extracellular medium. In addition, they are active players in cases of neuronal injury and/or under inflammatory conditions, contributing to the repair system and to the neuroinflammatory responses.

### **C. Synaptogenesis**

Synaptogenesis is the formation of synapses between neurons in the nervous system. Although it occurs throughout a healthy person's lifespan, an explosion of synapse formation occurs during early brain development, known as exuberant synaptogenesis (Huttenlocher & Dabholkar, 1997). This exuberant synaptic growth begins around twelve weeks after conception and continues until the eighth month after birth. Similarly, in mice it starts around ED 15 and continues until weaning (around PND20) (**Figure 1**). Synaptogenesis is particularly important during an individual's critical period, during which there is a certain degree of synaptic pruning due to competition for neural growth factors by neurons and synapses. Processes that are not used, or inhibited during their critical period will fail to develop normally later on in life (Comery et al., 1997).



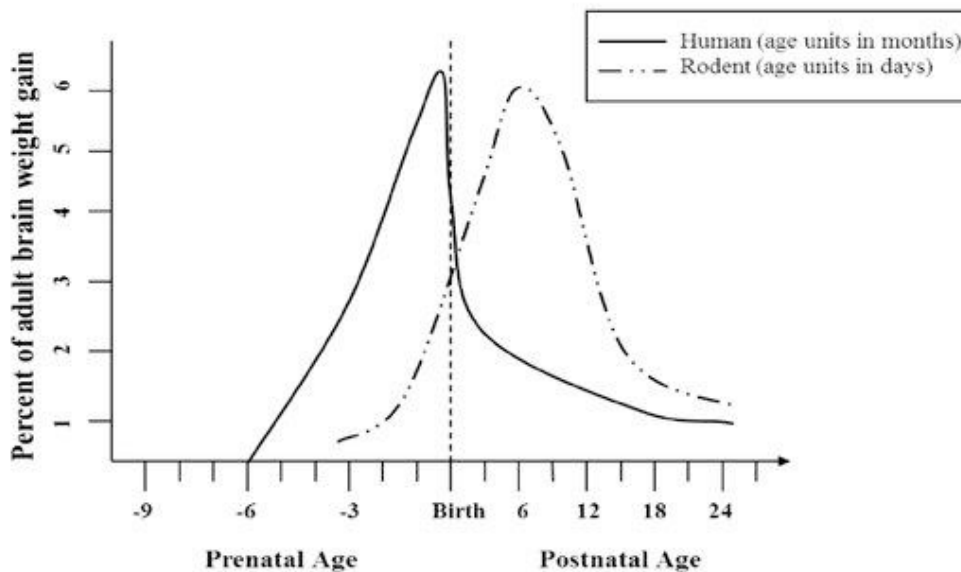
#### ***D. Brain Growth spurts***

Brain growth spurt (BGS), refers to periods of rapid growth and development in the brain that occur during specific periods in infancy, childhood, and adolescence. During these periods, the brain undergoes significant changes in its structure and function, including the formation of new neural connections and the pruning of unused connections.

The first major brain growth spurt occurs during the prenatal period, as the brain rapidly develops and forms new neurons and neural pathways. After birth, there are several additional growth spurts that occur during infancy and childhood, with one of the largest occurring during the first two years of life.

In the mammalian brain, a massive wave of synaptogenesis occurs during the brain growth spurt (Dobbing & Sands, 1979a) (**Figure 3**). Glial cells play a vital role in regulating synaptogenesis during these critical periods. Once an immature synapse is formed, astrocytic processes surround the synaptic cleft, creating a tripartite synapse that will allow effective neurotransmission in mature synapses. Additionally, astrocytes release cholesterol, which promotes the generation of spontaneous excitatory postsynaptic potentials (EPSCs) and enhances the strength of immature synapses (Chung et al., 2015; Farhy-Tselnicker & Allen, 2018). It is important to note that there is an excessive generation of synaptic connections during this embryonic period (Bourgeois et al., 1994; Huttenlocher & Dabholkar, 1997; Stiles & Jernigan, 2010). Astrocytes, along with oligodendrocytes and microglia, are known to play an indispensable role for proper synaptogenesis in the early development of neural circuits (Allen & Barres, 2009; Chung et al., 2015; Simons & Nave, 2016).

Another significant period of brain growth occurs during adolescence, when the brain undergoes significant changes in its structure and function, particularly in regions that are involved in decision-making, impulse control, and social behavior.



**Figure 3** – Brain growth spurt in humans and rodents. (Dobbing & Sands, 1979b)

Research has shown that disruptions to these periods of brain growth and development, such as from environmental toxins, malnutrition, or early childhood trauma, can have long-lasting effects on cognitive and emotional functioning (Heyer & Meredith, 2017; Philippot et al., 2016). Understanding brain growth spurts is therefore important for researchers and clinicians working to promote healthy brain development in children and adolescents.

## II. Neuro-inflammation

When it is about inflammation of the Central Nervous System (CNS), we refer to it as "neuro-inflammation." It was long believed that the CNS was protected from external aggressions by the blood-brain barrier (BBB). Today, there is no doubt about the existence of inflammatory processes within the CNS. Neuro-inflammation is characterized by the production of pro-inflammatory mediators within the CNS. These mediators are primarily generated and released by so-called "immune-competent" cells. While neurons and oligodendrocytes play a role in neuro-inflammation, the key players are microglial cells, previously mentioned, which are the resident immune-competent cells, along with astrocytes. Interest in neuro-inflammatory phenomena continues to grow due to their association with neurodegenerative conditions such as Alzheimer's disease or Parkinson's disease. This phenomenon is also strongly linked to the development of neurodevelopmental disorders such as Autism Spectrum Disorders (ASD) or schizophrenia. Although neuro-inflammation is often associated with damage to the CNS, it can also occur independently of pathological processes.

## 1. Neurogenesis and neuro-inflammation

Pro-inflammatory cytokines play a significant role in various processes involved in the development of the nervous system, such as synaptogenesis (Ben Achour & Pascual, 2010) and neurogenesis (Monje et al., 2003). The basal activity of pro-inflammatory cytokines contributes to homeostasis within the central nervous system (Glezer et al., 2007). During an inflammatory response, microglia and pro-inflammatory cytokines can have either a neuroprotective or neurotoxic effect. Interestingly, the receptors for pro-inflammatory cytokines are concentrated in areas implicated in learning and memory in the hippocampus (Parnet et al., 2002).

The quantity of activated microglia is negatively correlated with the survival rates of newly generated hippocampal neurons (Ekdahl et al., 2009). A study from 2003 demonstrates that certain pro-inflammatory cytokines are capable of reducing adult hippocampal neurogenesis (Monje et al., 2003).

### A. IL-1 $\beta$

IL-1 $\beta$  is an isomer of IL-1. In addition to the detrimental effects of activated microglia, pro-inflammatory cytokines also have individual effects on neurogenesis. Studies have revealed that IL-1 $\beta$  can lead to severe depletion of developing neuroblasts and alter the fate of neural stem cells in the SVZ (Wu et al., 2012). Another study demonstrated that when inflammation was reduced (via the anti-inflammatory properties of pioglitazone); IL-1 $\beta$  production was reduced, correlated with an increase in BDNF levels, and an improvement in neurogenesis (Das et al., 2019). BDNF is a growth factor involved in neuronal survival and also acts on the growth and differentiation of new neurons.

### B. IL-6

IL-6 is predominantly produced by astrocytes in the central nervous system (CNS) (Wagoner et al., 1999). This important cytokine is involved in microglial activity and inflammatory responses. Depending on the CNS microenvironment, IL-6 can exert either a neurotoxic effect or a neuroprotective effect (Kummer et al., 2021). For example, overexpression of IL-6 inhibits hippocampal neurogenesis (Monje et al., 2003). Similar data have been observed regarding the effect of IL-6 on neurogenesis in the SVZ (Shigemoto-Mogami et al., 2014).

## C. TNF- $\alpha$

The role and consequences of an excess of TNF- $\alpha$  are debated within the literature. This cytokine has been shown to be detrimental to hippocampal and SVZ progenitor cells (Bernardino et al., 2008). However, TNF- $\alpha$  enhances the proliferation of adult neural stem cells (Widera et al., 2006). Thus, depending on the receptors involved, the effect of TNF- $\alpha$  can be either neuroprotective or neurotoxic.

TNF- $\alpha$  has two types of receptors: tumor necrosis factor receptor 1 (TNF-R1) and tumor necrosis factor receptor 2 (TNF-R2). TNF-R1 induces a neurotoxic effect, while TNF-R2 induces a neurotrophic effect (Policastro et al., 2020). The difference between these two receptors is believed to stem from their molecular structure. TNF-R1 has a domain called the "death domain," which is not found in TNF-R2 receptors (Wardhani, 2022).

### 2. Neurodevelopment and neuro-inflammation : Maternal Immune Activation model

Prenatal environmental infections can be the root cause of neurodevelopmental and neuropsychiatric disorders that, despite their diversity, share pathological mechanisms. A growing body of evidence indicates that Maternal Immune Activation (MIA) during pregnancy is a significant risk factor for offspring developing neuropsychiatric disorders, mostly Autism Spectrum Disorders (ASD). Infections during pregnancy activate the mother's immune system, leading to various alterations in the fetal environment that can negatively impact offspring development (Sotgiu et al., 2020). Specifically, inducing MIA during critical periods of fetal neurogenesis can result in irreversible changes in brain structures and associated functions in the offspring. The relevance of inflammation in these disorders suggests a correlation between brain dysfunction and alterations in pro-inflammatory cytokines, as well as in the number and/or morphology of microglial cells (Cieřlik et al., 2020). Throughout prenatal inflammation, there are apparent links between ASD and the penetration of pro-inflammatory agents into the developing brain (Eissa et al., 2020). In vivo experimental data have indicated that male offspring from rats treated with lipopolysaccharide (LPS), which mimics a bacterial infection, exhibited communication deficits and repetitive behaviors reminiscent of autism-like behaviors (G. A. Lee et al., 2021). Furthermore, preclinical studies on prenatal infection and MIA animal models have shown abnormalities in the neuronal development of offspring, along with an

increase in microglial cells, as observed in schizophrenic patients (Purves-Tyson et al., 2021). It should be noted that the activation and increased number of microglial cells have also been demonstrated post-mortem in the cerebral cortex of patients with autism and schizophrenia (Edmonson et al., 2014). However, the cellular and molecular links between AIM-mediated disruptions in fetal brain development, brain dysfunction, and the onset of neuropsychiatric disorders are still unclear. Current knowledge suggests a combination of genetic, epigenetic, and environmental factors, along with their involvement in the dysregulation of neurotransmission (Masini et al., 2020).

### **III. Developmental Origin of Health and Disease (DoHAD)**

#### **1. General concept**

The early postnatal environment includes several essential elements, many of which are largely transmitted through mother-child interaction. The study on different animal models has shown that maternal bioactive agents can be transferred from the mother to her child via the placenta. They can influence brain development in children, potentially predisposing them to the development of certain pathologies in adulthood. These elements are the basis of Barker's theory of developmental origins of health and pathology, which advanced that the early life environment determines brain function and the development of pathologies in adulthood through programming early brain structure and function (Silveira et al., 2007). In addition, the consequences of early adversity on cognitive functions are also confirmed by preclinical studies in rodents (Loi et al., 2017).

The concept of developmental origins (Developmental Origins of Health and Disease) is based on the observation that the early environment (maternal and even paternal nutrition, chemical compounds, pollution, drugs, infections, lifestyle, etc.) has long-term effects on the phenotype of the individual. Epidemiological and experimental studies show the impact of an intrauterine growth retardation, but also of excess fetal weight, on the susceptibility to later develop metabolic syndrome, but also other pathologies (mental illnesses, osteoporosis, infertility, etc.). Epigenetic processes, which are involved in the regulation of gene expression profiles during development, serve as the molecular basis for these effects. These include DNA methylation, post-translational histone changes, and chromatin remodeling. By its plasticity, the epigenome integrates the impact of the environment and generates structural and/or

functional modifications of tissues during the inducing development, in adults, variability in response and adaptation to new environmental stimuli and susceptibility/resistance to developing certain diseases (Chavatte-Palmer et al., 2012). The term developmental plasticity proposed by some authors is defined as the ability of a single genotype to produce several different phenotypes in response to changes in environmental circumstances (Bateson et al., 2014), a process intimately involved in the development of various neuropathologies.

## 2. Neurodevelopmental pathologies

Neurodevelopmental disorders are a public health challenge due to complexity and heterogeneity of the etiology in conjugation with the high prevalence attained. In accordance with the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5; American Psychiatric Association, 2013), neurodevelopmental disorders (NDDs) are a collection of conditions that typically manifest in childhood and persist throughout life. NDDs encompass a variety of disorders characterized by atypical brain development, resulting in a wide range of symptoms that can affect cognition, communication, adaptive behavior, and psychomotor skills. Examples of NDDs include epilepsy, common monogenic neurological disorders (such as Fragile X and Rett syndrome), rare genetic disorders, intellectual disability, bipolar disorder, autism spectrum disorder (ASD), motor disorders (such as Tourette syndrome and obsessive-compulsive disorder), attention-deficit/hyperactivity disorder, and specific learning disorders (involving difficulties in reading, writing, and mathematics). Given the vast array of symptoms associated with NDDs, multiple factors have been implicated as potential causes, including genetic, environmental, infectious, and traumatic influences. These factors often interact with one another rather than acting independently.

Presently, a considerable number of genes associated with neurodevelopmental processes, including synaptic function and neuronal regulation at the transcriptional and translational levels, have been discovered (Widagdo & Anggono, 2018; Zoghbi & Bear, 2012). In fact, disruptions in over 1000 different genes that contribute to brain development and function have been observed in relation to neurodevelopmental disorders. This number continues to rise, indicating an incomplete penetrance of genetic risk factors. For instance, while it is estimated that monogenic situations account for approximately 25% of the disease, the

remaining 75% of autism spectrum disorder (ASD) cases present challenges in understanding their causes (Sarovic, 2021).

Throughout development, environmental factors can influence gene activity through epigenetic mechanisms like DNA methylation or histone modification (Roth & David Sweatt, 2011). Consequently, various maternal factors during pregnancy statistically elevate the risk of neurodevelopmental disorders in offspring. These factors include maternal obesity, diabetes, smoking, anxiety/depression, psychosocial stress, autoimmune disease, asthma, and infection. Each of these factors individually increases the risk of neurodevelopmental disorders in offspring by 30% to 80% (Maher et al., 2018). Furthermore, these factors are not specific to particular neurodevelopmental disorders; they exert comparable effects on conditions such as ASD, attention-deficit/hyperactivity disorder, Tourette syndrome, and others. It is likely that the risk of these disorders accumulates as multiple risk factors interact.

Therefore, it is plausible that the complex interaction between genetic and environmental factors, encompassing inflammatory and psychosocial pathways, particularly during the gestational period, influences the phenotypic manifestation of neurodevelopmental disorders (Woolfenden et al., 2022).

### 3. Neurodegenerative diseases

#### A. Parkinson's disease (PD)

Parkinson's disease (PD) is a neurodegenerative condition characterized by chronic, progressive, and multisystemic effects. It is influenced by various factors and has multiple possible causes. PD primarily affects individuals in the later stages of life. The likelihood of developing PD increases with age and reaches its highest point between 70 and 79 years old (Hirsch et al., 2016).

The primary cause of motor impairments in Parkinson's disease (PD) is the degeneration of dopaminergic (DA) neurons in the nigrostriatal system. It is estimated that up to 70% of DA cells in this system are lost before the cardinal motor symptoms of PD become apparent (Giguère et al., 2018). Neurogenesis, the process of generating new neurons, predominantly occurs during early nervous system development. In humans, DA neurons in the substantia nigra (SN) are formed between weeks 5 and 7 after fertilization, coinciding with the development of the enteric and olfactory nervous systems (Goldstein et al., 2013).

The concept of Developmental Origins of Parkinson's Disease (DOPD) suggests that early factors may contribute to the damage or disruption of DA neurons and their precursors. These factors could result in an acute reduction in the number of DA neurons. Consequently, as individuals age, the progressive loss of neurons, combined with this initial reduction, would lead to an insufficient number of neurons to maintain normal function (von Linstow et al., 2020). Alternatively, a prenatal factor could interfere with the normal developmental process, causing an accelerated loss of neurons throughout life and leading to subclinical alterations. In this scenario, some permanent cellular changes would occur. The DOPD hypothesis has been previously proposed by several authors (B. Barlow et al., 2007; Grova et al., 2019; Landrigan et al., 2005; Schwamborn, 2018; Tartaglione et al., 2015a).

## **B. Amyotrophic lateral sclerosis (ALS)**

Amyotrophic Lateral Sclerosis (ALS), also known as Charcot's disease or Lou Gehrig's disease, is a chronic progressive disease of the central nervous system. According to Orphanet, the incidence (approximately 1/50,000 per year) and prevalence (approximately 1/20,000) are relatively uniform in Western countries. These parameters are higher in certain islands in the Pacific. This disease has a slight male predominance with a male-to-female ratio of approximately 1.5:1.

ALS is characterized by involvement of motor neurons in both the central (brain) and peripheral (spinal cord) nervous system, affecting the part of the nervous system that controls voluntary movement. The disease is characterized by a weakening of skeletal muscles, gradually leading to paralysis of the limbs, respiratory muscles, and those responsible for speech and swallowing.

While the majority of cases are of environmental origin (sporadic forms), 5-10% are of genetic origin (familial forms). For a long time, only one gene whose mutation is responsible for the disease was known: the *SOD1* gene. In its non-mutated form, the enzyme coded by this gene neutralizes oxygen-free radicals that are deleterious to cells. Mutations in *SOD1* found in ALS patients result in a modification of the enzyme's structure, which then becomes toxic. Since then, about thirty other implicated genes have been identified. Among them, the *TARDBP* gene contributes to about 4% of familial cases and 1% of sporadic cases. This gene codes for the TAR DNA-binding protein 43 (TDP-43). This protein is involved in regulating the transcription of DNA



into messenger RNA and is normally located in the nucleus of cells (Suk & Rousseaux, 2020). But in ALS, it is sometimes found only in the cytoplasm. This phenomenon is even a marker of the disease in the majority of patients, especially those with sporadic ALS or associated with frontotemporal dementia (Tiloca et al., 2013). Numerous studies are therefore aimed at understanding the influence of the loss of TDP-43 function in the nucleus and/or its toxic function in the cytoplasm.

Finally, even though motor neurons are the cells that degenerate in ALS, glial cells and also immune cells from the systemic contribute to the disease. Indeed, studies conducted in animal models of ALS have highlighted a chronic inflammatory state in which astrocytes, microglial cells, and macrophages present in the environment of motor neurons play a deleterious role and contribute to the progression of the disease (Gerber et al., 2012; Gomes et al., 2019).

#### 4. Environmental Origins

There is growing evidence that environmental factors can play a significant role in the development of neurodevelopmental and neurodegenerative diseases. These factors include exposure to toxins, such as lead and mercury, as well as pesticides, air pollution, and certain infections (Nabi & Tabassum, 2022).

In the case of neurodevelopmental disorders, such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD), studies have suggested that exposure to certain toxins during pregnancy or early childhood can contribute to their development (Pugsley et al., 2022). For example, maternal exposure to air pollution has been associated with an increased risk of ASD in offspring (Chun et al., 2020). Additionally, prenatal exposure to pesticides has been linked to a higher likelihood of developing ADHD (Manley et al., 2022).

In terms of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, there is evidence to suggest that exposure to environmental toxins may play a role in their development. For example, exposure to heavy metals, such as lead and mercury, has been linked to an increased risk of developing Alzheimer's disease (M. El-Sewify et al., 2022). Additionally, exposure to pesticides has been associated with an increased risk of developing Parkinson's disease (Gamache et al., 2019). Concerning ALS, this neurodegenerative disease is more and more considered as a multifactorial disease, subject to the influence of genetics and the environment. Regarding environmental factors, several factors are suspected of

contributing to the onset of the disease or accelerating its progression. Among them, exposure to pesticides (Guardia-Escote et al., 2021; Sánchez-Santed et al., 2016), consumption of mushrooms (Lagrange et al., 2021), or exposure to cyanotoxins such as BMAA (Newell et al., 2022a) are suggested.

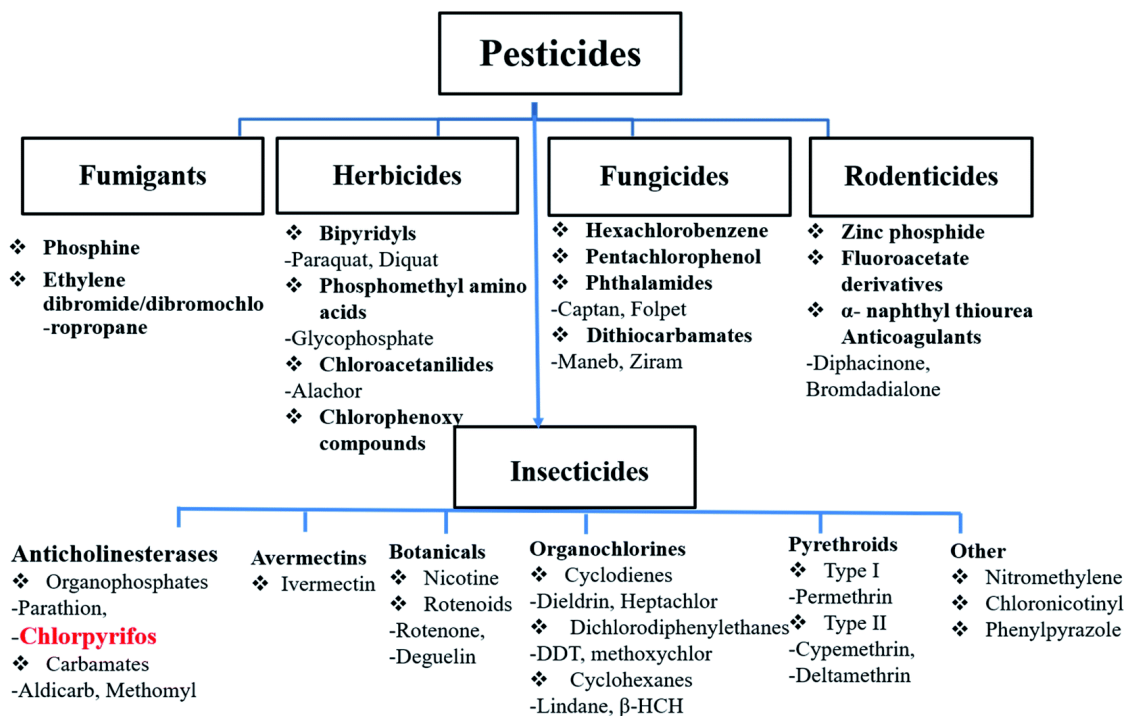
So, in the following sections of this manuscript, certain environmental factors will be addressed, starting with pesticides and herbicides, followed by toxins naturally produced by our environment, particularly by cyanobacteria.

## Chapter 2: Environmental Origin Factors in neurological disorders

### I. Pesticides

#### 1. Definition and classification

The word pesticide comes from the prefix “pest” meaning harmful (animal or plant origin) and the suffix “-cide”, from the Latin meaning “slaughter, kill”. A pesticide is therefore a substance used to control organisms considered harmful. This includes, among others, herbicides, insecticides and fungicides (Figure 4).



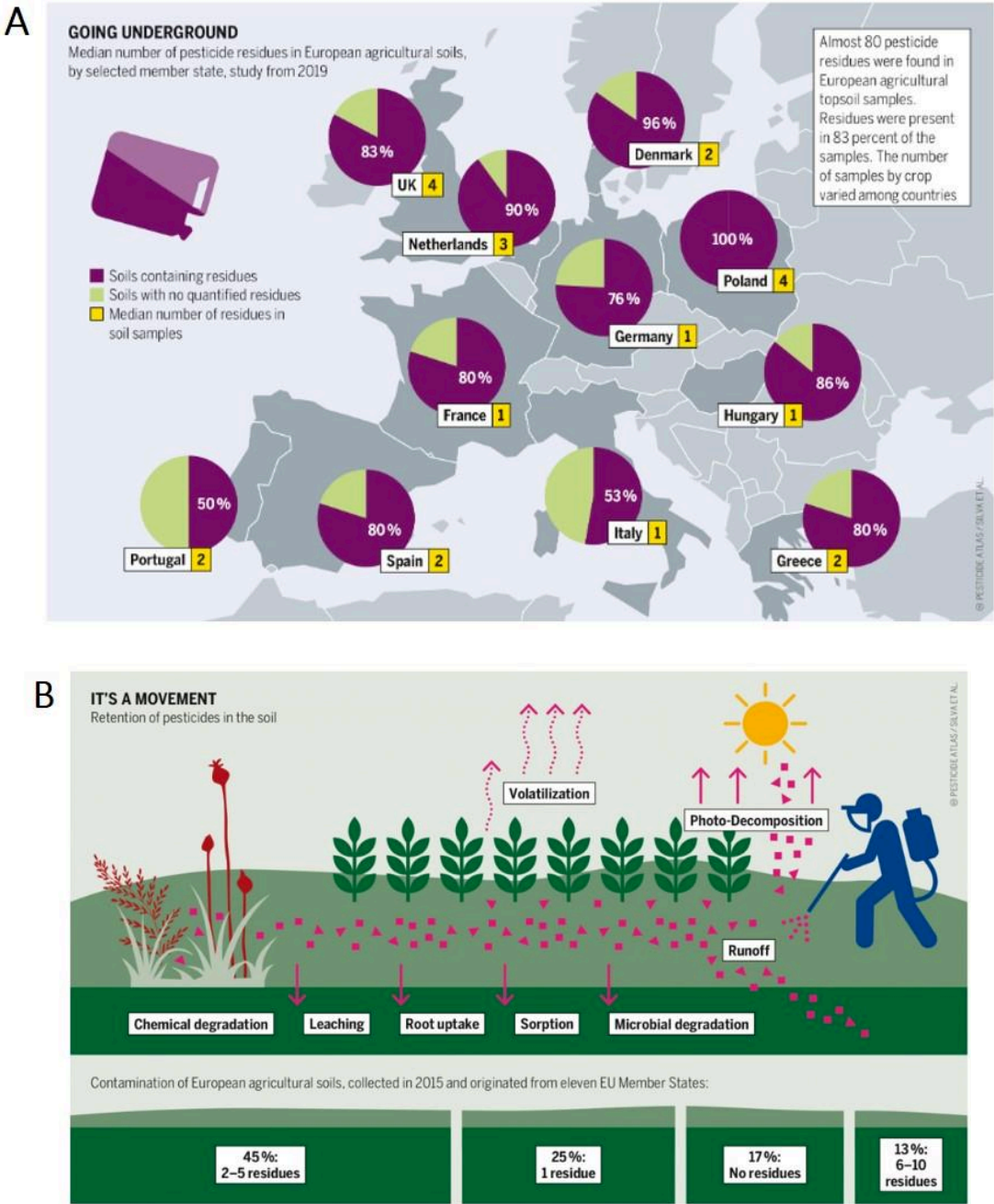
**Figure 4** - Pesticides classification based on their targets and the chemical structure. (Nandhini et al., 2021)

Considering the definition given by the Food and Agriculture Organization (FAO), pesticides are “any substance or mixture of substances (synthetic or natural) intended for preventing, destroying or controlling any pest, including vectors of human and animal disease, and unwanted species of plants or animals causing harm” (Hernández, 2023).

Conventional agriculture currently relies on the widespread use of pesticides to control various pests (like insect, fungi, and “weeds”) and thus ensure crop yields to meet the needs of the constantly growing population.

The widespread and intensive use of pesticides in agriculture leads to contamination of soils and other environmental resources through different ways including, root uptake, microbial

degradation or runoff. In Europe, soil analyses revealed that more than 80 percent of 317 agricultural topsoils tested contained pesticide residues. The persistence of pesticide residues in soils is a major threat to soil living organisms that are important to the right ecological balance (Figure 5).



**Figure 5** – (A) Number of pesticide residues in European agricultural soils from a study conducted in 2019. (B) Contamination ways of European agricultural soils, collected in 2015 from 11 EU Members sates. (Pesticide, n.d.)

Recently, the European Food Safety Authority (EFSA) has highlighted the lack of standardized methods for evaluating the eco-toxicological effects of pesticides on soil microorganisms. In addition, some parameters are rarely assessed, such as chronic effects, medium- and long-term effects, but also use of lower concentrations/doses.

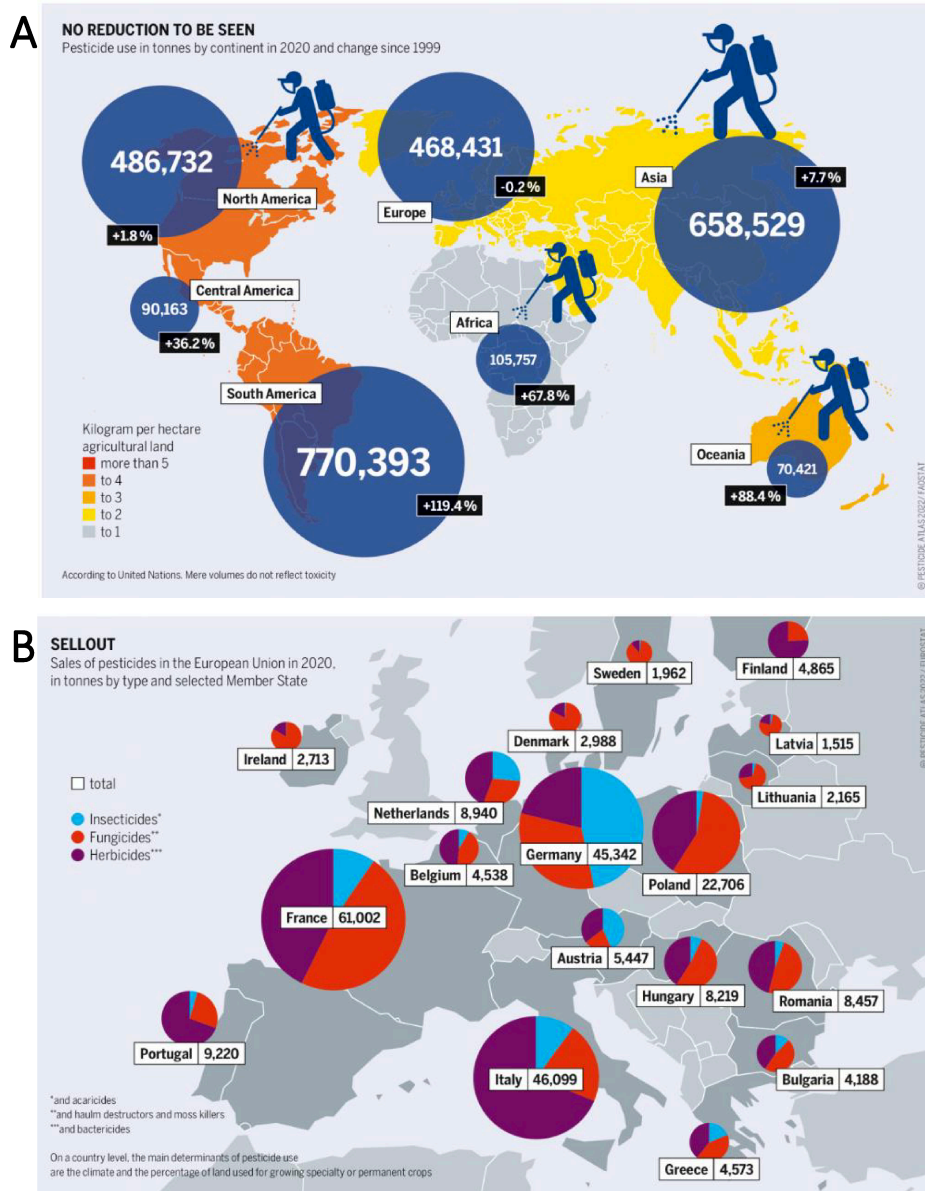
The Herbicide Resistance Action Committee (HRAC) classifies herbicides into 23 groups and subgroups according to their specific biochemical modes of action, with a specific designation for each group. This classification enables the establishment of strategies for preventing the development of specific resistances to each mode of action. In 2020 this classification was revised, and is currently based on a numerical system. It can be defined three major groups of herbicides:

- Herbicides affecting cell metabolism
- Herbicides affecting photosynthesis
- Herbicides affection growth and cell division

## 2. Pesticide use in France and around the world

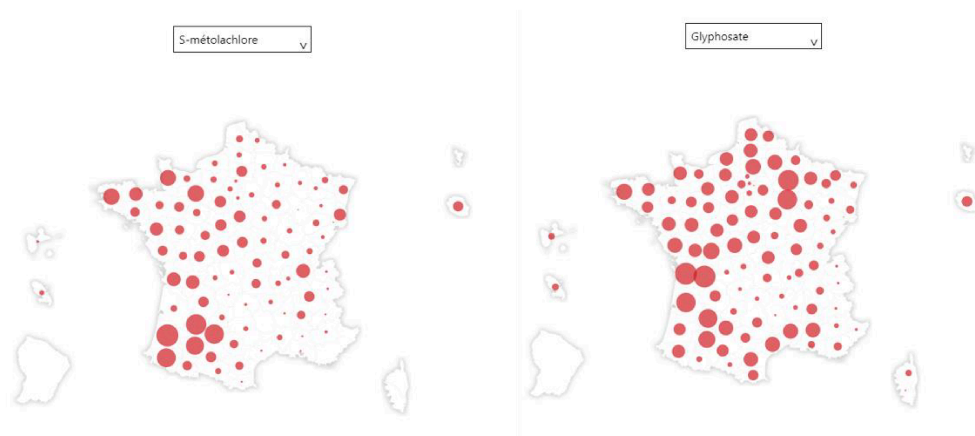
Europe is the world's third-largest consumer of pesticides, behind the South and North Americas and Japan. For more than 20 years, the use of these phytosanitary agents has increased by 2- 20%. In France, an average of 75,000 tons of pesticides are produced every year and almost 65,000 tones are used, which represents a third of European consumption (**Figure 6**).

As the leading agricultural producer in Europe, France is also the leading consumer of pesticides, with 90% for agricultural use (against 10% used for communities or individuals). In 2018, sulphur was the main active substance sold in France, followed by glyphosate.



**Figure 6**– Worldwide pesticides use in tons by continent in 2020 (A) and sales of pesticides in the European Union in 2020 in tons by type and selected member states Number of pesticide residues in European agricultural soils from a study conducted in 2019. (B) (Pesticide, n.d.)

To monitor the use of this kind of products, the government uses the NODU indicator (Number of Dose Units). It reports the quantity sold of each active substance at a “unit dose”, corresponding to “the maximum dose of that active substance applicable to an average treatment in a given year”. In 2018, substances that contributed the most to the NODU were S-Metolachlor (herbicide), glyphosate (herbicide) and cypermethrin (insecticide) (**Figure 7**).



**Figure 7** – Cartography of the number of dose unit of S-Metolachlor and Glyphosate in France in 2018

The World Health Organization (WHO) has established a classification system that distinguishes between the most and least dangerous forms of certain pesticides according to their dangerous impact hazards to human health. It shall consider the toxicity of the active ingredient.

Since March 20<sup>th</sup>, 2015, glyphosate has been classified as "probably carcinogenic" by the International Agency for Research on Cancer (IARC). In 2017, the European Chemicals Authority (ECHA) restated that glyphosate was not carcinogenic, mutagenic or reprotoxic. However, in their public report "Pesticides and Health", INSERM believes that the link between exposure to glyphosate and the occurrence of certain pathologies is highly underestimated.

Currently, glyphosate is still allowed in France, since its use has been reintroduced for 5 more years, following the vote of EU members in 2017. This was not the case for BASTA F1, which the active substance is glufosinate ammonium, which was withdrawn from its Marketing Authorization Application (MAA) by the French Agency for Food, Environmental and Occupational Health & Safety (ANSES). The Agency considers that "risks to human health from exposure to glufosinate cannot be ignored, including risks to the health of persons applying the product or likely to be in a space where it was applied".

Though, its use is still very important in other European countries or in the world, as it shows its effectiveness against glyphosate resistant species (Dong et al., 2020a).

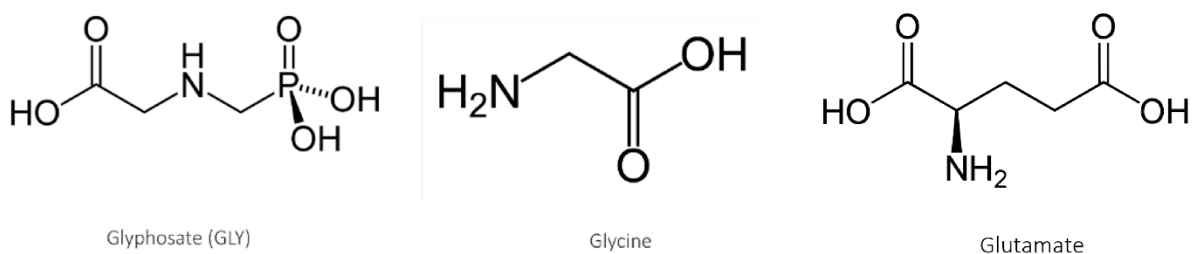
### 3. Glyphosate (GLY)

Glyphosate is the active ingredient contained in commercially available herbicides. It is a synthetic molecule discovered in the 50s by the Swiss chemist Henri Martin. First produced exclusively by Monsanto in the 70's (now Monsanto/ Bayer), under the name of Roundup®, it has been in the public domain since 2020. Other formulations have then emerged, such as Attila® (Phileual) or Barbarian® (Barclay Chemicals). All these products are commonly called Glyphosate Based Herbicide (GBH).

GBH contains, in addition to the active substance, surfactants such as sulphuric or phosphoric acid. These strengthen the action of the herbicide by increasing its water solubility helping its absorption by plants. Thus, in toxicological assessment, it needs to distinguish the action of the active substance from that of the adjuvants.

#### A. Main target

Glyphosate (N-(phosphonomethyl) glycine, C<sub>3</sub>H<sub>8</sub>NO<sub>5</sub>P) is a weak organic acid. It is a structural analogue of a natural amino acid, glycine. It has a phosphonate group (the name comes from the contraction of "glycine" and "phosphonate") (**Figure 8**).



**Figure 8** – Molecular structure of glyphosate and his structural analogues glycine and glutamate

Glyphosate is a systemic, non-selective and post-emergence herbicide. An herbicide is systemic when is absorbed and translocated by the plant. Thus, the active compound penetrates the plant through its leaves to reach the sap. It will be then conveyed from one organ to another to the roots. It therefore has a generalized action and will destroy the whole plant. An herbicide is non-selective and post-emergence when it acts on weeds or already germinated grasses. All categories of sprayed plants are destroyed.



## B. Herbicide molecular action mechanism

The action of glyphosate is made through the inhibition of enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS). This enzyme belonged to the Shikimate pathway, involved in the biosynthesis of aromatic amino acids. This pathway is mainly located in chloroplasts (Figure 9).

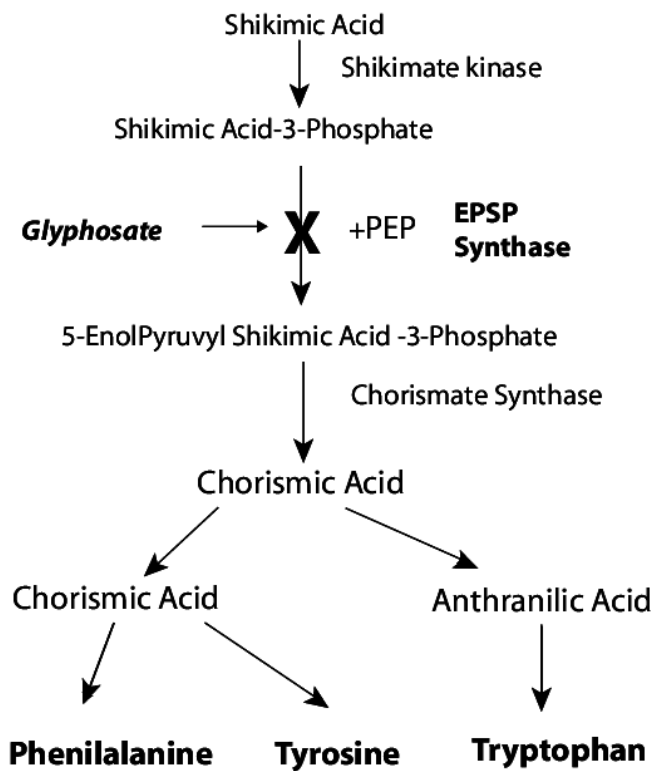


Figure 9 – Glyphosate mechanism of action

Glyphosate induces a decrease in the synthesis of aromatic amino acids, such as phenylalanine, tyrosine and tryptophan. These amino acids are involved in the proteins and chlorophyll synthesis, various plant hormones and secondary metabolites. As they are essential for the plant, glyphosate action leads to necrosis and death of the plant. However, this pathway does not exist in mammal or other animal fields.

## C. Neurotoxic effects

Glyphosate has long been considered as an “eco-friendly” compound as no negative impacts were observed on organisms other than plants (Williams et al., 2000). Indeed, it was assumed that human cells did not possess the “shikimate pathway”; a biological pathway

present in plants and negatively impacted by glyphosate (Samsel & Seneff, 2015a). However, even if this enzyme is not present in vertebrates, an increasing number of studies suggest that glyphosate can lead to impairments also in Humans. Indeed, previous studies have shown that our «intestinal flora» possesses, like plants, shikimate pathway allowing the supply of essential amino acids, such as Tryptophan, Tyrosine or Phenylalanine. Interestingly, other biologically active molecules such as serotonin, dopamine or noradrenaline depend on metabolic precursors of this pathway (Samsel & Seneff, 2015a).

#### a. Epidemiological studies and toxicological value

In a toxicological study, there are several standard endpoints for assessing the toxicity of a compound. To assess acute toxicity, two parameters are determined: Lethal Dose 50 (LD 50) and Lethal Concentration 50 (LC 50). The LD50 is the dose at which 50% of the dose-receiving organisms do not survive. Similarly, the lethal concentration 50 (LC50) is the concentration at which 50% of the organisms that inhaled or ingested do not survive.

To assess chronic toxicity, the NOAEL and LOAEL can be evaluated. The NOAEL (No Observed Adverse Effect Level) is the maximum concentration or quantity of a substance that does not induce any detectable adverse effects in an exposed population. The Lowest Observed Adverse Effect Level (LOAEL) is the lowest dose (or concentration) of exposure in an experiment that produces an observed adverse effect. The relationship between all these parameters is shown in Figure 10.

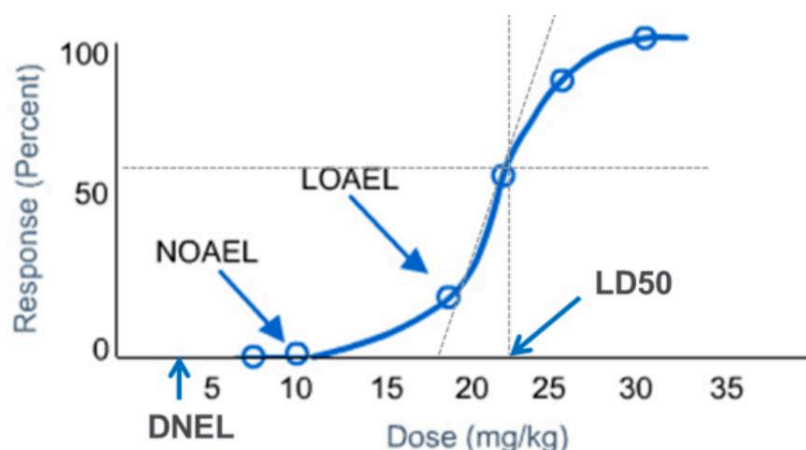


Figure 10 – Relationship between LD50, LOAEL, NOEL on a typical dose response curve

A value of LD50 or NOAEL is dependent on various factors such as the species but also the route of administration (dermal, oral, through inhalation). In rats, the oral LD50 is > 5000 mg/kg body weight (bw), whereas via the dermal route the value is > 2000 mg/kg bw. By inhalation, also in rats, the LC50 after a 4H exposure is > 5mg/L (inhaled air). Based on the results of various animal studies, glyphosate has low acute toxicity. Under the World Health Organisation (WHO) classification system, glyphosate is listed in Group III (low toxicity). Comparably, based on different studies in rats and rabbits, a NOAEL of 50 mg/kg/day bw could be determined (Pesticide and Health Effect).

In epidemiological studies, indicators of acute or chronic intoxication are well described. Cases of suicide by ingestion have been recorded. A study in Asia of 131 patients reported clinical and biological signs following ingestion of glyphosate. The most common symptoms are dysfunction of the digestive and respiratory systems as well as kidney and liver dysfunction (H. L. Lee et al., 2000; H.-L. Lee et al., 2000).

A growing number of studies suggest that glyphosate is neurotoxic. Indeed, epidemiological studies suggest that pesticides are particularly involved in the occurrence of sporadic cases of neurodegenerative diseases such as Parkinson's or Alzheimer's (B. K. Barlow et al., 2005; Gallegos et al., 2016a; Patel et al., 2006). According to the Environmental Protection Agency (EPA), over 36% of cases of acute glyphosate poisoning involve neurological symptoms (Samsel & Seneff, 2015a). Because of the multiple means of exposure to GLY (dermal and/or ocular contact, ingestion of treated cultures or contaminated water, inhalation of the product), the risk of developing problems, especially neurological ones, is increasing. Interestingly, it appears that acute or chronic exposure to GLY induces the development of Parkinsonian syndrome (Barbosa et al., 2001; G. Wang et al., 2011). In 2001, Barbosa and her team reported the case of a 54-year-old man who accidentally sprayed himself with glyphosate and a month later developed Parkinsonian syndrome. Later, Wang and colleagues (2011) reported a 44-year-old woman (without any history) with Parkinsonian syndrome following chronic exposure to glyphosate. She had a stiffness, a slowness of gestures as well as tremors at rest, at the 4 limbs but without reaching her short-term memory. These observations led to various experimental studies, evaluating the effects of exposure to glyphosate under *in vitro* and *in vivo* conditions.

## b. Experimental studies

Various studies were conducted to determine the behavioral, cellular and molecular effects of exposure to glyphosate. It is known that glyphosate and his metabolites could pass through the blood-brain barrier (BBB) and also affect it (Martinez & Al-Ahmad, 2019). Previous studies have shown that it could induce neuroinflammation, especially caused by an increase of oxidative stress leading to an over-activation of glial cells (Astiz et al., 2012).

Another study in mice showed that the maximum permissible glyphosate concentrations recognized by the competent authorities led to a significant decrease of cell migration and differentiation of NSCs. In addition, an increase in expression of the gene coding for oxidative stress was observed. These results indicate that glyphosate does have a neurotoxic effect, particularly during nervous system development (Masood et al., 2021).

Moreover, because of its structural analogy with glycine and glutamate, it can influence synaptic transmission. It is important to note that glutamate is the main excitatory neurotransmitter of Central Nervous System (CNS). Glycine is a major inhibitor neurotransmitter involved in regulating the activity of NMDA glutamate receptors. Thus, Cattani and colleagues have demonstrated that exposure to Glyphosate Based Herbicides (GBH) induces over-activation of NMDA receptors inducing an increase in Ca<sup>2+</sup> inflow and leading to oxidative stress and neuronal cell death (Cattani et al., 2014). These cellular and molecular disturbances are the source of phenotypic and behavioral disturbances.

Species	Dose and Exposition	Time Exposition	Objectives	Results	References
Swiss mice	Roundup®: 250 or 500 mg/kg/day orally	Subchronic exposition: 6 weeks Chronic exposition: 12 weeks	Assess the effects of acute or repeated GBH exposure on the developing brain of young and adult mice	Chronic/subchronic exposure: - ↓ locomotion - ↑ anxiety, depressive behavior - ↓ 5-HT immunoreactivity  Chronic exposure: ↓ TH immunoreactivity	(Ait Bali et al., 2017a)
Swiss mice	Roundup®: 250 or 500 mg/kg/day orally	Subchronic exposition: 6 weeks Chronic exposition: 12 weeks	Evaluate the effects of GBH on learning and memory functions, AChE activity, and oxidation/antioxidation homeostasis	Chronic/subchronic exposure: - Impaired recognition and retention memory - ↓ AChE activity - ↓ SOD and peroxidase activity  Chronic exposure caused impairment in working memory	(Bali et al., 2019)
Swiss mice	Roundup®: 250 or 500 mg/kg/day orally	From GD0 to PND21	Evaluate the behavioral (PND5-PND25) and biochemical (PND60) effects of gestational and lactational exposure to GBH on offspring	- Delayed sensorimotor development from PND5 to PND25 - ↓ locomotion, anxiety, ↓ sociability, cognitive impairment - ↓ number of TH <sup>+</sup> cells - ↓ AChE activity - Activation of microglia and astrocytes. ↑ TNF-α expression and ↓ BDNF. ↑ TrkB levels	(Ait-Bali et al., 2020a)

Species	Dose and Exposition	Time Exposition	Objectives	Results	References
Wistar rats	GLY: 24 or 35 mg/kg intraperitoneally	Dams received injections every 48 h from GD8 to GD20, totaling seven injections over two weeks	Evaluate the neurobehavioral effects of GLY in neonate rats after gestational exposure	<ul style="list-style-type: none"> <li>- Delay in the development of neonatal reflexes in offspring</li> <li>- ↓ locomotion</li> <li>- Learning and memory deficits</li> <li>- Negative regulation of the Wnt5a/CaMKII pathway</li> </ul>	(Coullery et al., 2020a)
Sprague-Dawley rats	GLY, Roundup®: 5 mg/kg/day orally	From GD10 to PND22	Compare the potential effects of a low dose of GLY and GBH on maternal behavior and maternal neuroplasticity, focusing on the hippocampus and cingulate gyrus	<ul style="list-style-type: none"> <li>- Initial reduction in maternal licking behavior, followed by a subsequent increase</li> <li>- Impairment of neurogenesis and plasticity in the mother's hippocampus</li> <li>- Alteration in synaptophysin expression</li> </ul>	(Dechartres et al., 2019)

**Table 1** – Summary of *in vivo* effects of glyphosate and its commercial formulation in rodents (Costas-Ferreira et al., 2022)

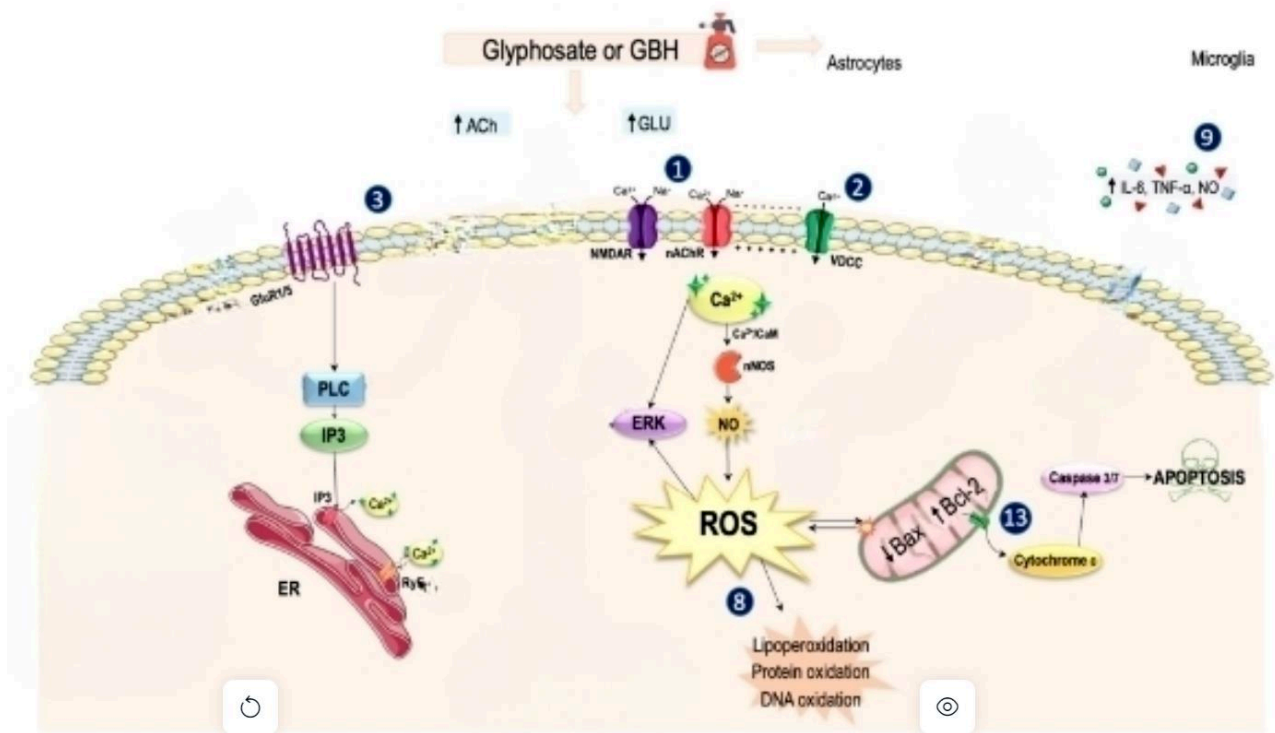
Many studies in rodent evaluating exposure to glyphosate or GBH produces have been conducted. Main effects including changes in brain development and in the neurotransmission systems, as well as oxidative stress and neuroinflammation could be observed. These processes lead to death of the neuron and finally to the appearance of behavioral changes. Few of them are listed in **Table 1**. Most of these studies show the neurotoxic effects of glyphosate administered at early ages during the intrauterine period and lactation, although chronic or acute exposure in adulthood also causes important alterations in the function and structure of the nervous system.

The previously mentioned alterations could be implicated in the delay in sensorimotor development observed after GBH exposure (Ait-Bali et al., 2020a). Ait Bali and co-workers have shown GBH exposure caused a delay in the appearance of sensorimotor reflexes and a deficit in the motor development of the offspring. Similarly, de Oliveira and her team showed that maternal exposure to 50 mg/kg of Glyphosate Based Herbicide (GBH) induced behavioral impairments and oxidative stress in offspring 's with neurodevelopmental disorders (de Oliveira et al., 2022a). Rat studies have shown that new-borns (pups) may be exposed during gestation and lactation, and this exposure induces alteration of brain enzymes in the mother and offspring (Cattani et al., 2014). Other studies have pointed out that chronic or sub chronic exposure during the perinatal period, juvenile or adulthood induces decreased of locomotor activity, emotional behaviors and anxiety in pups; possibly related to impaired GABAergic, dopaminergic and/or serotonin systems (Ait Bali et al., 2017a; Ait-Bali et al., 2020a; Coullery et al., 2020a; Gallegos et al., 2016a). Furthermore, an increase in anxiety levels and depression-like behaviour of the animals was also observed (Ait Bali et al., 2017a; Ait-Bali et al., 2020a). These alterations in mood could come from the decrease in serotonin and noradrenaline levels.

It has been shown exposure to glyphosate during the perinatal period not only affects offspring behaviour but also maternal behaviour. These changes in maternal behaviour could lead to the development of behavioral modifications in the offspring, like social behaviour, in an early way and later in adulthood (Ait-Bali et al., 2020a).

### c. *Neuro-molecular mechanism in vertebrate*

Glyphosate as well as GBH act on various targets to generate the deleterious effects observed in many studies over the literature. It would reduce the absorption and glutamate metabolism by glial cells. In addition, it induces an increase of glutamate release in the synaptic slit. This excessive level of extracellular glutamate results in an excitotoxicity. As a result, there is a binding to the NMDA receptors (**Figure 11 -1**) and the VDCC-type voltage-dependent calcium channels (**Figure 11 -2**), either by the excess of glutamate present in the synaptic slit or directly by glyphosate due to its structural analogy. It leads to a massive intake of  $\text{Ca}^{2+}$  into cells resulting in an increase of oxidative stress via ROS production (**Figure 11 -8**). This important intracellular  $\text{Ca}^{2+}$  concentration results also from the activation of the GluR1/5 metabotropic receptors, which via a signaling cascade induces the release of  $\text{Ca}^{2+}$  by the endoplasmic reticulum (ER) (**Figure 11 -5**). Excessive levels of intracellular ROS leads to cell death by apoptosis (**Figure 11 -13**). Activation of glial cells is also possible leading to the release of pro-inflammatory cytokine like IL-6 or  $\text{TNF}\alpha$  (**Figure 11 -9**).



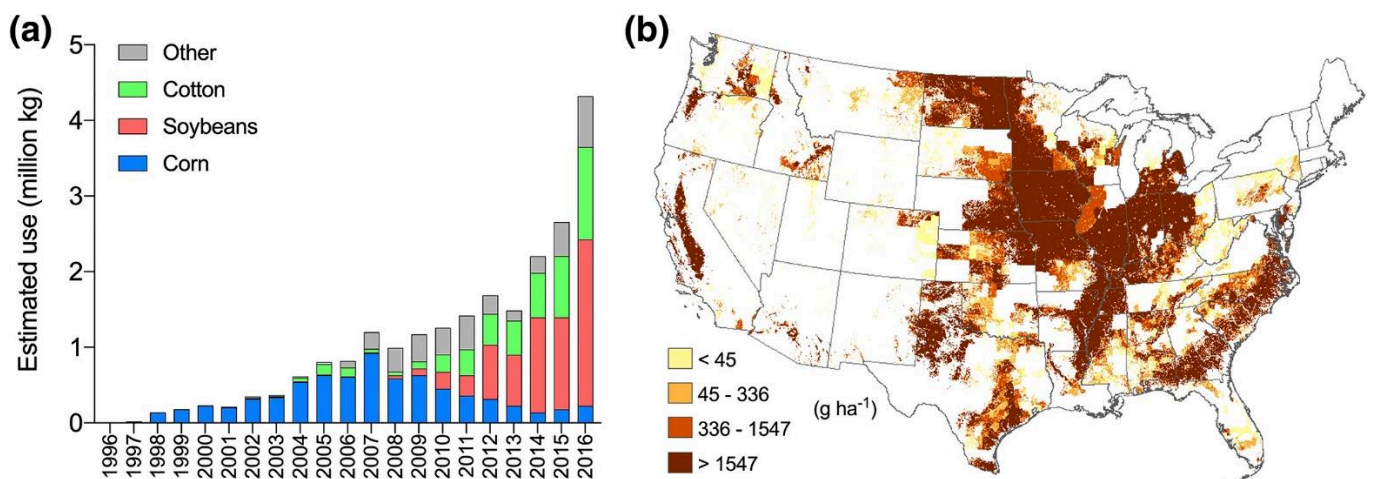
**Figure 11** – Possible in vivo mechanism of action of glyphosate (or Glyphosate Based Herbicide) in the nervous system (Costas-Ferreira et al., 2022)

Interestingly it has been shown that glyphosate promote high proliferation of cyanobacteria bloom trough eutrophication phenomenon, making cyanobacteria particularly resistant to this compound (W. Wang et al., 2021). Therefore, the combine use of GLY and Ammonium Glufosinate (GLA), on other well-known herbicides, has been proposed in attempt to counteract the plant resistance to GLY (Ganie & Jhala, 2017a; Samsel & Seneff, 2013a, 2015a).

#### 4. Glufosinate Ammonium (GLA) or Phosphinotrycin (PPT)

Glufosinate Ammonium (GLA) or Phosphinotrycin (homoalanin-4-yl (methyl) phosphinic acid, or PPT) is the active ingredient of a non-selective, broad-spectrum, low-systemic organophosphate herbicide. It is a “contact” herbicide with a foliar penetration from the base to the tip of the leaf. In the 1970s, the German Hoechst Group developed it and this substance was gradually introduced worldwide between 1980s and 1990s, first in Japan, then in the United States (US) and the United Kingdom.

GLA is the active ingredient of many commercial formulations such as Basta F1<sup>®</sup>, Rely<sup>®</sup> or Liberty Link<sup>®</sup>. It was developed as a foliar herbicide to control a wide variety of weeds. In addition, it is the first herbicide recommended for the annual treatment of vineyards (REF 43 JFV). All over the world, approximately 12 million hectares / year crops in 2014 have been treated with GLA notably linked to a considerable increase use in the US over the past decade (Figure 12) (Busi et al., 2018)



**Figure 12** – Estimated uses of GLA in United States from 1996 to 2016. (a) Estimated use of GLA by type of crop. (b) Representative map of agricultural use of GLA in the US. (Takano & Dayan, 2020)

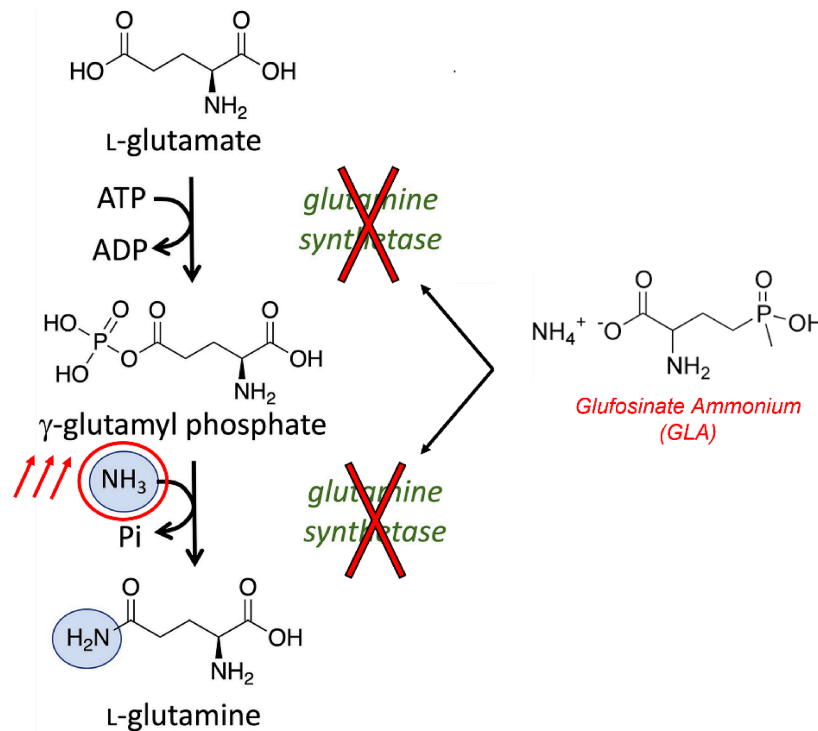
The use of GLA has increased in the recent decades, mainly to counter the increasing number of glyphosate-resistant weeds, as GLA works as an alternative herbicide to glyphosate, the most popular herbicide in the world. This herbicide is currently registered in more than 40 countries worldwide (Babalola et al., 2021). In France, GLA was commercialized by Bayer as Basta F1<sup>®</sup>. However, since October 24, 2017, ANSES has withdrawn its marketing authorization (MA) as risks to the health of farmers and people around treated sites were not negligible due to its reprotoxic nature.

### A. Main target and molecular mechanism in plant

This non-selective herbicide acts as a powerful, competitive, non-reversible inhibitor of Glutamine Synthetase (GS). This enzyme is essential for the detoxification and regulation of amino acids in plants (Calas et al., 2008). GS is responsible for the uptake of ammonia in plants through the synthesis of glutamine from glutamate. Thus, GLA, through the inhibition of this



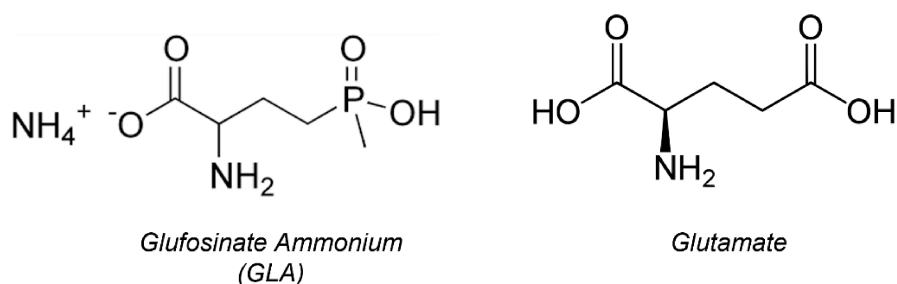
enzyme, induces the accumulation of ammonia ( $\text{NH}_3$ ) and a deficiency of several amino acids resulting in the death of the plant (**Figure 13**) (Miflin et al., 1981).



**Figure 13** – Glufosinate mechanism of action. Synthesis pathway of glutamine from glutamate via the action of Glutamine Synthetase in 2 steps. (Takano & Dayan, 2020)

## B. Neurotoxic effects

GLA is a structural analogue of Glutamate (**Figure 14**). As said previously, it is a competitive inhibitor of GS, a key enzyme produces not only in plants but also in almost all animal species that have been studied (Calas et al., 2008). GS is present in many organs, including the CNS, where it plays a key role in regulating the metabolism of glutamate (Schousboe et al., 2014). The GS is found exclusively in glial cells, capable of transforming glutamate into glutamine. Thus a defect in the activity of GS can lead to neurotoxic effect related to  $\text{NH}_3$  triggering various disturbances such as an impairment of the BBB or in astrocyte morphology (Cooper & Plum, 1987).



**Figure 14** – Molecular structure of Glufosinate Ammonium and his structural analogues glutamate

### a. Epidemiological studies and toxicological value

As previously mentioned about GLY, various standard toxicological parameters related to GLA exposure have been determined based on specific studies. On previous GLA studies, LD50 (acute toxicity) and NOAEL (chronic toxicity) values were calculated for different species and administration mode. The main values most found in the literature are presented in the below **Table 2**.

Species	Type of toxicological studies	Toxicological endpoints	Values
<b>Rat</b>	Acute – Oral	LD50	1510 mg/kg bw
	Acute – Dermal	LD50	> 4000 mg/kg bw
	Chronic – Carcinogenic	NOAEL	2,1 mg/kg/day bw
	Chronic – Developmental	NOAEL	10 mg/kg/day bw
<b>Mice</b>	Acute – Oral	LD50	431 mg/kg bw
	Chronic – Carcinogenic	NOAEL	10,8 mg/kg/day bw
<b>Rabbit</b>	Acute – Dermal	LD50	> 2000 mg/kg bw
	Chronic – Developmental	NOEAL	6,3 mg/kg/day bw

**Table 2** - Acute and chronic GLA toxicological values by animal species (Wolterink et al., 2012)

Many cases of accidental poisoning or suicides have been recorded in Human. The main clinical signs associated with GLA intoxication are respiratory insufficiency, circulatory failure, and moderate to severe neurotoxicity (Mao et al., 2011; Watanabe & Sano, 1998). Considering different cases of GLA ingestion, symptoms are firstly gastrointestinal disorders followed by, in a second time, by neurological symptoms such as convulsions or anterograde amnesia associated with lesions of selected brain structures (hippocampus, striatum). Toxicological data from Japanese GLA intoxicated patients showed respiratory failure and unconsciousness associated with convulsion, possibly related to GS inhibition and increased glutamate (Calas et al., 2016).

Alarmingly, in 2011, a study revealed the presence of 3-[hydroxy(methyl) phosphinoyl] propionic acid (MPPA), a GLA metabolite, in blood samples from non-pregnant and pregnant women but also in the umbilical cord (Aris & Leblanc, 2011). These findings suggest that early exposure to environmental pollutants highlights the importance of considering the consequences of perinatal exposure to GLA, even in the absence of apparent symptoms, in relation to the concept of the "pregnancy exposome". Within our laboratory, this notion has been largely studied through various aspects.

#### **b. *Experimental studies***

In an adult mouse model, acute intraperitoneal (IP) GLA exposure at 75 mg/kg induced epileptic episodes. Following an intracerebroventricular injection, convulsions are also observed. These seizures would be caused by the massive production of nitric oxide (NO) through the activation of NMDA receptors. It also has been shown that an acute exposure to GLA disrupt glutamatergic transmission (Lapouble et al., 2002; Nakaki et al., 2000).

A chronic administration of moderate doses of GLA (5-10 mg/kg) in a mice model induces changes in adult learning, memory properties, attention and anxiety disorders. At the cerebral level, a change in astroglial activity and disruption of glutamatergic homeostasis has been observed (Calas et al., 2008; Meme et al., 2009). The deleterious effects of GLA exposure during earlier stages and especially during development have also been shown. Previous studies conducted in our laboratory have shown that a perinatal exposure to low-dose of GLA (0.2-1 mg/kg) induced seizures and autistic-like behaviour in offspring. In addition, it induces SVZ neurogenesis disruption as well as an increase of the oxidative stress (Feat-Vetel et al., 2018;

Herzine et al., 2016; Laugeray et al., 2014). A more recent study by Dong and colleagues shows a decrease in locomotor activity and autistic-like behaviour in offspring following a maternal exposure to low-dose of GLA (2 mg/kg) (Dong et al., 2020b).

### c. Neuro-molecular mechanism in vertebrate

All these clinical and experimental data on GLA show the importance of the glutamatergic homeostasis. Indeed, it seems that the glutamatergic system is a preferential target for exposure to environmental agents such as pesticides.

GLA as well as Glufosinate based herbicide act on various targets to generate the deleterious effects observed in many studies over the literature.

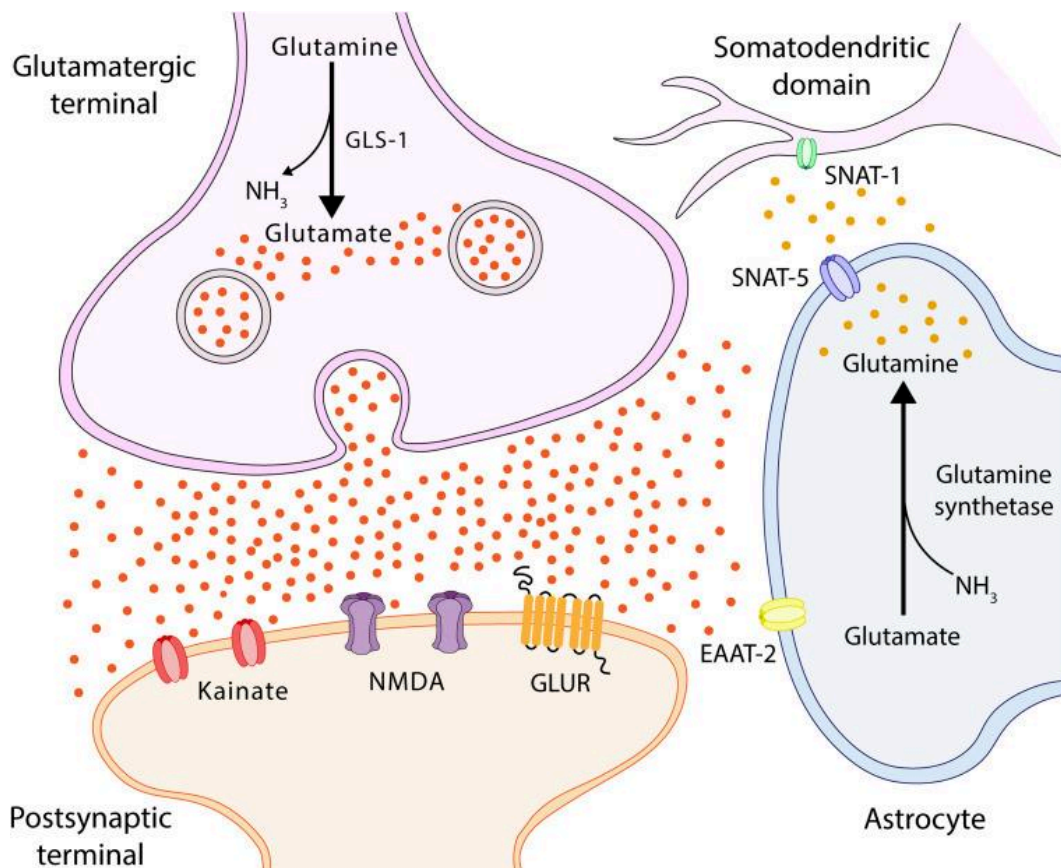


Figure 15 – Tripartite glutamatergic synapse.

Due to its structural analogy with glutamate, any system and signaling pathway involving glutamate are potentially target by direct or indirect action of GLA. Glutamate is involved in many cellular processes and presents in many organs like brain where it is considered as the main neurotransmitter of the CNS. It is found in the tripartite synapse, presynaptic vesicles containing glutamate and in the various receptors and enzymes recycling glutamate (Figure 15).

Glutamate also plays an important role in cytoskeleton stabilization via polyglutamylation mechanism, essential for the proliferation, migration and cell differentiation regulation. It is also involved in various metabolic pathways, especially in mitochondria. Thus, like GLY, GLA can disrupt all mechanisms involving glutamate. It can thus generate oxidative stress following a significant increase of intracellular ROS due to the hyper stimulation of metabotropic receptors (mGluR1/5) and NMDA receptors (Faro et al., 2013).

In addition, as mentioned earlier, GLA acts on GS, an enzyme notably present in the CNS, where it is mainly found in astrocytes (Feat-Vetel et al., 2018). One of the primary roles of astrocytes is to protect neurons against excitotoxicity by taking up excess ammonia and glutamate and converting it into glutamine via the enzyme GS. Through its inhibitory action, GLA exposure may induce changes in astroglial function, which can affect neuronal functions. GS also plays a key role in regulating neurotransmitter metabolism in the brain, catalyzing the synthesis of glutamine from ammonia and glutamate (**Figure 15**). Glutamine is then used as a substrate for the synthesis of neurotransmitters such as glutamate and aminobutyric acid (GABA) (Suárez et al., 2002). Interestingly, studies have shown that when it is absorbed, GLA can break down into ammonia and glufosinic acid. Ammonia can affect the nervous system by causing tissue irritation and inflammation, while glufosinic acid can act as an inhibitor of GABA, an important inhibitory neurotransmitter in the brain (Isaacson & Scanziani, 2011).

## II. Micro-alga and cyanobacteria

### 1. General concept

The term “microalgae” generally refer to microscopic algae. These organisms can be unicellular or multicellular and are typically capable of photosynthesis. Microalgae can be found in various environments such as oceans, lakes, rivers, wet soils, and even tree barks. Within microalgae, there are two main types: eukaryotic organisms and prokaryotic organisms (**Table 3**). Among eukaryotes, there are several different species including dinoflagellates and diatoms. These species are mainly found in marine environments. About prokaryotic organisms, it includes all cyanobacteria which are mainly found in freshwater and brackish waters. Cyanobacteria are gram-negative prokaryotic organisms appeared more than 3.5 billion years ago (Whitton & Potts, 2002). Formerly called "blue-green algae," this name was given to them

because they possess a pigment, phycocyanin, which allows them to carry out oxygenic photosynthesis and gives them their bluish colour.

	Cyanobacteria	Dinophyceae (dinoflagellates)	Bacillariophyceae (diatoms)
<b>Cell type</b>	Prokaryotes	Eukaryotes	Eukaryotes
<b>Pigment</b>	Chlorophyll a , Phycobilines (i.e. phycocyanin)	Chlorophyll a and c2, xanthophyll (i.e. diadinoxanthin)	chlorophyll a, c1, c2 and c3, (i.e. fucoxanthin, diatoxanthin,
<b>Cell Wall</b>	Peptidoglycans	Cellulose	Silica
<b>Morphology</b>	Unicellular Filamentous	Unicellular with 2 flagella	Pinnate (bilateral symmetry) Centric (radial symmetry)
<b>Reproduction</b>	Asexual	Asexual & sexual	Asexual & sexual
<b>Average species number</b>	6280	2294	Up to 100 000

**Table 3** - Morphological and cytological characteristics for cyanobacteria, dinoflagellates and diatoms (Réveillon, 2015)

Regarding eukaryotic dinoflagellates and diatom species, they are believed to have appeared later due to endosymbiosis, especially with cyanobacteria species providing them chloroplasts necessary for photosynthesis (Bhattacharya et al., 2004). Microalgae and cyanobacteria have several advantages and can be used in many fields. For example, they can be used in the food industry for their high nutrient content. They can also be used as biofuels or to purify wastewater (Sathasivam et al., 2019).

Cyanobacteria are very important for aquatic ecosystems as they constitute a major source of nutrients and oxygen through their photosynthetic action. As primary producers, they constitute the first link in the aquatic food chain. Under favorable conditions, cyanobacteria can form visible colonies, called "blooms." For this, a high concentration of nutrients (nitrogen and phosphorus), sufficient brightness, and sufficient residence time in the water are required to achieve high growth. This corresponds to the phenomenon of eutrophication and accentuates "bloom" formation. Indeed, cyanobacteria have the particularity of exploiting excessive inputs of nutrients, especially nitrogen and phosphorus. The excessive presence of these "blooms" can lead to a significant imbalance in the aquatic ecosystem, which can have a

significant environmental and health impact. (Nugumanova et al., 2023). In fact, more than 200 species of microalgae and cyanobacteria are believed to be harmful. Indeed, certain species possess the ability to produce secondary metabolites with harmful biological activity: toxins (Facey et al., 2022).

This is a metabolite produced by cyanobacteria with a harmful biological activity in a given environment or system. Cyanotoxins could be classified in different groups according to their structure or functional property (i.e. hepatotoxin, neurotoxins, dermatotoxin and cytotoxins). These toxin-producing species can be found everywhere but with variations depending on the type. For example, toxic dinoflagellate species could be found in marine environments, while cyanobacteria produce most their toxins in freshwater (Metcalf et al., 2021). In France, the predominant cyanobacterial species that could be found is *Microcystis aeruginosa* (hepatotoxins) or *Planktothrix rubescens* and *Dolichospermum spp* (neurotoxins). In the early 21st century, it was estimated that 95% of cyanobacteria were capable of producing  $\beta$ -N-methylamino-L-alanine (BMAA) (Cox et al., 2018). In the following section, characteristics and environmental and health effects associated with exposure to this contaminant will be discussed.

## 2. $\beta$ -N-Methylamino-L-alanine (BMAA)

### A. Environmental sources

#### a. Vegetal sources (cycas seeds)

Some plants are known for their ability to produce non-protein amino acids. Canavanine, for example, is found in legumes or seeds (Mardani-Korrani et al., 2021). However, their identification can sometimes be complex due to symbiotic phenomena that some plants can have with cyanobacteria (Kaasalainen et al., 2012; Rasmussen & Nilsson, 2003).

In the 1960s, BMAA was identified in the seeds of *Cycas* (Banack & Cox, 2003). Today, *Cycas micronesica* is considered an endangered species caused by various parasites (Marler et al., 2010). However, other species such as *Cycas micronesica revoluta* and *Cycas debaoensis* can produce BMAA (McCarron et al., 2014). It is interesting to note that BMAA isomers (DAB and AEG) have also been detected in *Cycas debaoensis* (McCarron et al., 2014). The presence of DAB has also been reported in numerous angiosperm species as well as in certain aquatic plants (Banack et al., 2010; Spáčil et al., 2010).

These observations show that BMAA and its isomers can also be produced by many plant species, some of which are known to have endosymbiotic cyanobacteria. The presence of BMAA and its metabolites within these organisms is therefore subject to controversy.

### b. Cyanobacteria

As previously mentioned, several species of cyanobacteria are capable of producing secondary metabolites. Among these, a large majority of cyanobacterial species are capable of producing BMAA (Cox et al., 2005). Generally, cyanobacterial toxins are produced in response to stress in an environment under extreme conditions. It has been shown that several factors are involved in the production of these toxins, including temperature, light intensity, growth phase, and nitrogen concentration in the medium.

The presence of BMAA in symbiotic cyanobacterial species such as *Nostoc sp* (Figure 16), isolated from the roots of *Cycas micronesica*, was the origin of renewed interest in BMAA in the early 2000s (Banack & Cox, 2003). This team hypothesized that BMAA production could be a common feature of cyanobacteria. Indeed, among the 41 selected strains of screened cyanobacteria, 95% were found to be capable of producing this toxin. However, variations in concentration have been reported depending on the species studied and the method used (Cox et al., 2005; Lage et al., 2016).

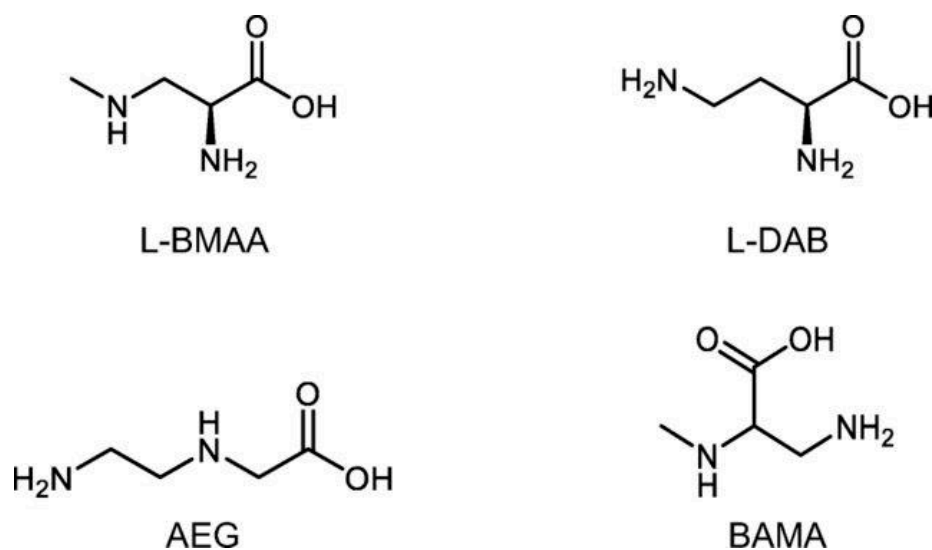


**Figure 16** – Picture of *Cycas micronesica* (A), (B) their coraloid roots, and (C) a root section revealing a green ring corresponding to the endosymbiotic cyanobacterium. (A. C. G. Chang et al., 2019)

With the use of new, more selective analytical techniques, conflicting results have been obtained since 2008 regarding the presence of BMAA. While many species have been examined, some studies have reported an absence of BMAA (Faassen et al., 2012). This finding has led to discussions on the analytical methods and the nature of the molecules measured in previous studies (Krüger, et al., 2012). Despite the validation of more sensitive analytical



methods for the detection and quantification of BMAA, no recent examination of a large number of cyanobacteria has been conducted. However, two species have been confirmed as producers of BMAA (i.e., *Nostoc* sp. PCC 7120 and *Leptolyngbya* sp. PCC 73110), but at relatively low concentrations (i.e., <1 µg.g<sup>-1</sup> DW) (Berntzon et al., 2013a; Jiang et al., 2014a). Recently, the analysis of two strains of coccal cyanobacteria isolated from the Mediterranean sponge *Petrosia ficiformis* (*Cyanobium* sp. ITAC108 and *Synechococcus* sp. ITAC107) revealed for the first time the presence of BMAA and microcystin in these organisms (Pagliara et al., 2021a).



**Figure 17** - β-N-Methylamino-L-alanine (BMAA) and its isomers 2,4-diaminobutyric acid (DAB), N-(2-aminoethyl)glycine (AEG), and β-aminomethyl-L-alanine (BMAA)

Regarding BMAA isomers, DAB has been observed several times while AEG has only been reported in a few strains (Jiang et al., 2014a; McCarron et al., 2014). However, the production of BMAA by all cyanobacteria has been called into question, while DAB and AEG have been detected more frequently in recent years (**Figure 17**).

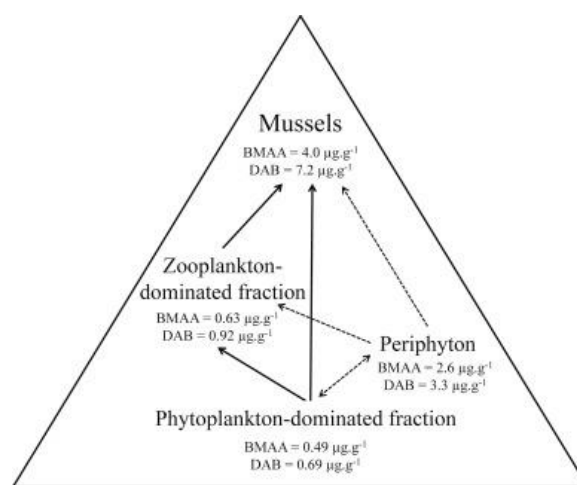
Furthermore, BMAA production is highly dependent on context. Its production will differ depending on environmental parameters during sampling or genetic variability. Moreover, among strains of the same species, production can vary, which is why not all strains of the same species necessarily produce BMAA or one of its isomers.

### c. Micro alga

Globally, no less than 175 different species of microalgae that are toxic to humans or harmful to marine biodiversity have been identified. Just like cyanobacteria, the presence of

microalgae leads to the closure of bathing areas. In France, many regions are affected by the worrying presence of microalgae, such as certain regions in Brittany or the Thau lagoon in the south of France. Several families of microalgae that produce toxins are monitored, including *Dinophysis* (diarrhetic toxin), which is the most widespread species.

In 2016, Réveillon and his team highlighted an increase in BMAA and one of its isomers, DAB, between lower and higher trophic levels within the ecosystem of the Thau lagoon (Réveillon et al., 2015a). This supports the hypothesis that the bioaccumulation of BMAA and DAB occurs through feeding processes, in this case, the mussels in the Thau lagoon (**Figure 18**) (Réveillon et al., 2015a).



**Figure 18** - Hypothesized bioaccumulation pathway within the Thau lagoon ecosystem. The plain arrows represent the hypothesized trophic links while dotted arrows represent non-confirmed links. (Réveillon et al., 2015b)

However the phytoplankton community is not dominated by cyanobacteria, but rather by microalgae such as diatoms or dinoflagellates.

Diatoms are a group of algae representing 40% of primary production in the ocean today (Main et al., 2016; Murch et al., 2004a). Due to their abundance in the marine phytoplankton community, particularly in nutrient-rich areas of the world's oceans, diatoms probably represent up to 20% of the world's photosynthetic carbon fixation (Glover et al., 2014). The production of toxins by diatoms was first reported in the 1980s when it was discovered that the marine and estuarine diatom genus *Pseudo-nitzschia* produced domoic acid, a neurotoxic amino acid. Until recently, this neurotoxic substance was considered the only toxin produced

by diatoms, but several studies have shown the production of BMAA and its isomers by marine diatoms (Jiang et al., 2014a; Lage et al., 2016; Réveillon et al., 2015a).

Dinoflagellates belong to freshwater and marine plankton. Some species are chlorophyll-containing, while others that are devoid of photosynthetic pigments feed on plankton. Some, like *Nocticula milaris* and *Noctulica scintillans*, can be bioluminescent when disturbed at night. Blooms can be deadly for the species present. Indeed, upon their death, these microalgae species release ammonia.

	Species	Isomères			References
		BMAA	AEG	DAB	
	Cyanobium sp.	X		X	(Pagliara et al., 2021b)
	Leptolyngbya sp.	X	X	X	(Jiang et al., 2014b)
<b>Cyanobacteria</b>	Nostoc sp.	X		X	(Banack et al., 2007; Berntzon et al., 2013b)
<b>Dinoflagellea</b>	Gymnodinium catenatum				(Lage et al., 2014a)
	Heterocapsa triquetra	X	X	X	
	Achnanthes sp.	X	X	X	(Jiang et al., 2014b)
	Coscinodiscus granii	X			(Lage et al., 2014a)
	Thalassiosira sp.	X	X	X	(Jiang et al., 2014b; Réveillon, 2015)
<b>Diatoma</b>	Aulacoseira sp.	X	X	X	(Violi et al., 2019)
	Chaetoceros socialis	X		X	(Réveillon et al., 2015b)
	Cyclotella sp.	X		X	(Violi et al., 2019)
	Fragilaria sp.		X	X	(Violi et al., 2019)
	Navicula sp.	X	X	X	(Jiang et al., 2014b; Violi et al., 2019)

Phaeodactylum tricornutum	X		X	(Lage et al., 2014a; Réveillon et al., 2015b)
Skeletonema sp.	X	X	X	(Jiang et al., 2014b)
Tabellaira sp.	X	X	X	(Violi et al., 2019)

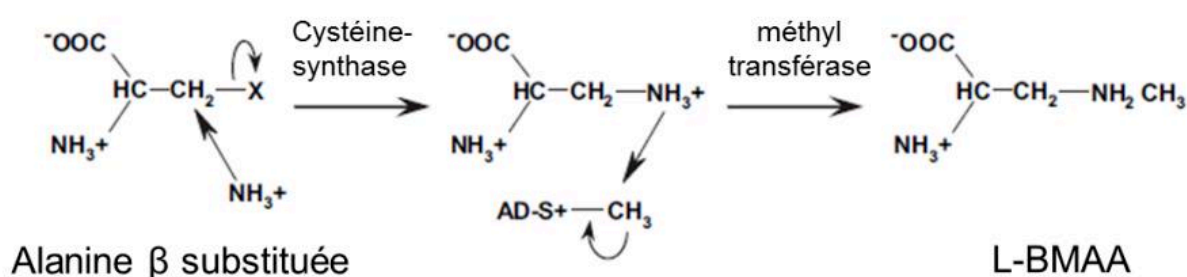
**Table 4** – List of species of microalgae (prokaryotic and eukaryotic) confirmed as producers of BMAA and its isomers.

About ten strains of diatoms and dinoflagellates have been identified as producers of BMAA and its isomers (Jiang et al., 2014a; Lage et al., 2014b). All species producing BMAA and its isomers are listed in **Table 4**. Only reliable and selective detection methods have been referenced.

## B. BMAA production and biosynthesis pathway

### a. Biosynthesis pathway

So far, identifying the functions and biosynthetic pathway of BMAA in cyanobacteria has remained difficult. However, in 2003 it was proposed that the synthesis of BMAA depended on 2,3-diaminopropionic acid (DAP), one of its secondary metabolites. This non-proteinogenic amino acid is synthesized from S-O-acetylsérine, phosphoserine, cysteine or 3-cyanoalanine, and ammonium. The synthesis of BMAA involves a cysteine-synthase enzyme as well as a methyl transferase to successively transfer an NH<sub>3</sub> group onto a substituted beta-alanine, and then a methyl onto the amine of the formed metabolic intermediate (**Figure 19**).



**Figure 19** – Biochemical pathway of BMAA biosynthesis proposed by (Brenner et al., 2003)

In 2022, a team attempted to confirm or refute hypotheses regarding the biosynthetic pathway of BMAA, using bioinformatics tools. Through the evaluation of 130 cyanobacteria genomes, they were able to assess the presence or absence of key enzymes involved in its

synthesis through six known potential metabolic pathways. The results of this study indicate that cyanobacteria can synthesize DAP, the precursor of BMAA, via the *SbnA* and *SbnB* genes. However, it is also possible that cyanobacteria can synthesize BMAA through pathways that remain unknown at this time (Mantas et al., 2022).

To this day, the biosynthetic processes of BMAA and its isomers have not been fully understood. While the strains of cyanobacteria that produce BMAA are not universally agreed upon, it is particularly noteworthy that studies have shown the presence of BMAA in the diatoms *Thalassiosira sp.* and *Phaeodactylum tricornutum* through genome sequencing, sparking particular interest in studying its biosynthesis (Amin et al., 2012; Bowler et al., 2008).

### **b. BMAA role**

The strong presence of BMAA in cyanobacteria suggests a crucial role, given their appearance on Earth over three billion years ago. From an ecological standpoint, BMAA can provide an ecological functional advantage as well as physiological improvement. Various studies have identified the different ecological and physiological roles of BMAA for cyanobacteria :

**Role in nitrogen metabolism:** Nitrogen availability plays an essential role in the production of BMAA by cyanobacteria (Downing et al., 2011; Monteiro et al., 2017), especially in an inter-specific competition between cyanobacteria species. Thus, BMAA has the ability to inhibit the activity of nitrogenase produced by nitrogen-fixing cyanobacteria. By limiting the growth and reducing this population, this creates leaves space and oxygen availability for non-nitrogen-fixing cyanobacteria populations. In 2020, Zhang and Whalen hypothesized that cyanobacteria have physiological adaptations that allow them to bioaccumulate BMAA below a threshold concentration in their organelles to avoid toxic effects (Zhang & Whalen, 2020). This could explain the coexistence of nitrogen-fixing and non-nitrogen-fixing cyanobacteria in the same environment, despite their joint ability to produce BMAA.

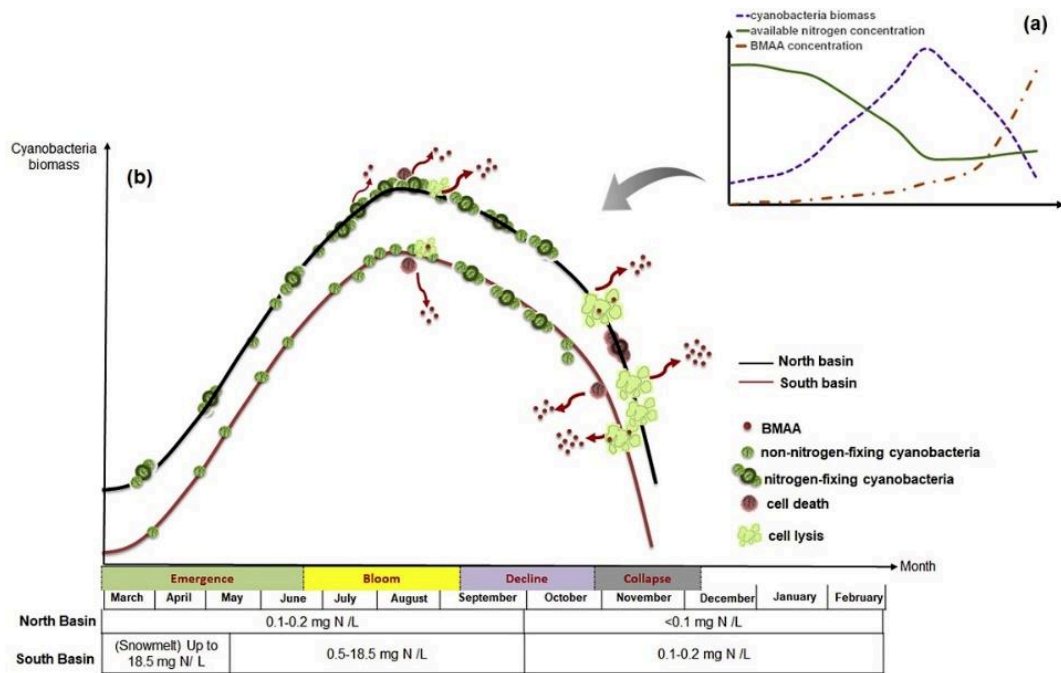
**Role in population control:** Furthermore, recent research has shown that in two diatom species (*Phaeodactylum tricornutum* and *Thalassiosira pseudonana*), BMAA acts as a toxic metabolite capable of controlling microalgal growth by triggering oxidative stress (Kim et al., 2022a). The same team also showed that DAB (an isomer of BMAA) would act as a defensive secondary metabolite (S.-Y. Kim et al., 2022).

Role in regulation of photosynthesis: Cyanobacteria are photosynthetic organisms. Therefore, they must protect their photosynthetic apparatus from photo-oxidative damage. A previous study suggests that BMAA would have a beneficial physiological effect on cyanobacteria present in the desert. Indeed, its role in reducing photosynthetic activity would reduce the amount of reactive oxygen species generated by strong light intensities. Thus, BMAA would have a role in photo protection under certain conditions, maintaining the metabolic integrity of cyanobacteria (Downing et al., 2015).

### *c. Factors influencing BMAA production*

In order to determine the factors favoring the production of BMAA, some research teams have cultivated strains of micro-algae, including cyanobacteria. In 2011, Downing and his team proposed a hypothesis on why detection of BMAA is difficult (Downing et al., 2011). In fact, in the laboratory, cyanobacteria are often grown in nitrogen-free environments while its detection is easier in samples from the environment (Jiang et al., 2014a).

Today, it is recognized that under nitrogen-deficient conditions, diazote-fixating and no fixative cyanobacteria (N<sub>2</sub>) can synthesize BMAA (Monteiro et al., 2017). However, in a nitrogen-limited environment, N<sub>2</sub> no fixative cyanobacteria produce more BMAA than those that bind diazote (Monteiro et al., 2017; Zhang & Whalen, 2020). Based on surveys conducted in Canada, a dynamic proposal between BMAA production and cyanobacteria blooms was modelled (**Figure 20**).



**Figure 20** - Hypothesized relationship among available nitrogen concentration, cyanobacteria biomass and  $\beta$ -N-methylamino-l-alanine (BMAA) production (a) and relationship between BMAA production and development of cyanobacterial harmful algal blooms composed of the non- $N_2$ -fixing cyanobacteria and  $N_2$ -fixing cyanobacteria in the North basin and South basin of Lake Winnipeg, Canada (based on CyanoHABs dynamics reported by Manitoba Sustainable Development and Environment and Climate Change Canada (2012, 2016) (b)

Hypothesized relationship among available nitrogen concentration, cyanobacteria biomass and  $\beta$ -N-methylamino-l-alanine (BMAA) production (a) and relationship between BMAA production and development of cyanobacterial harmful algal blooms composed of the non- $N_2$ -fixing cyanobacteria and  $N_2$ -fixing cyanobacteria in the North basin and South basin of Lake Winnipeg, Canada (based on CyanoHABs dynamics reported by Manitoba Sustainable Development and Environment and Climate Change Canada (2012, 2016) (b).

Thus, one of the major factors contributing to the abundance of micro-algae is the increase in anthropogenic activities. The one that seems to have the biggest impact is eutrophication. This involves an increased release of nutrients, particularly phosphorus and nitrogen, into water bodies. This increase in nutrients can lead to a significant proliferation of potentially harmful algae in lakes, rivers and marine waters. Recently, global warming has been shown to contribute to the development of cyanobacteria (Hecht et al., 2022; Lürling et al., 2018).

The United States Environmental Protection Agency (EPA) has summarized the main factors affecting algal blooms:

- Salinity
- High levels of dioxide
- The level of precipitation
- Sea level rise

Numerous studies have shown that in proliferative episodes, cyanobacterial agents can produce toxins to varying degrees, leading to a posing risk to the exposed populations (Rastogi et al., 2015).

### C. Neurotoxic effects

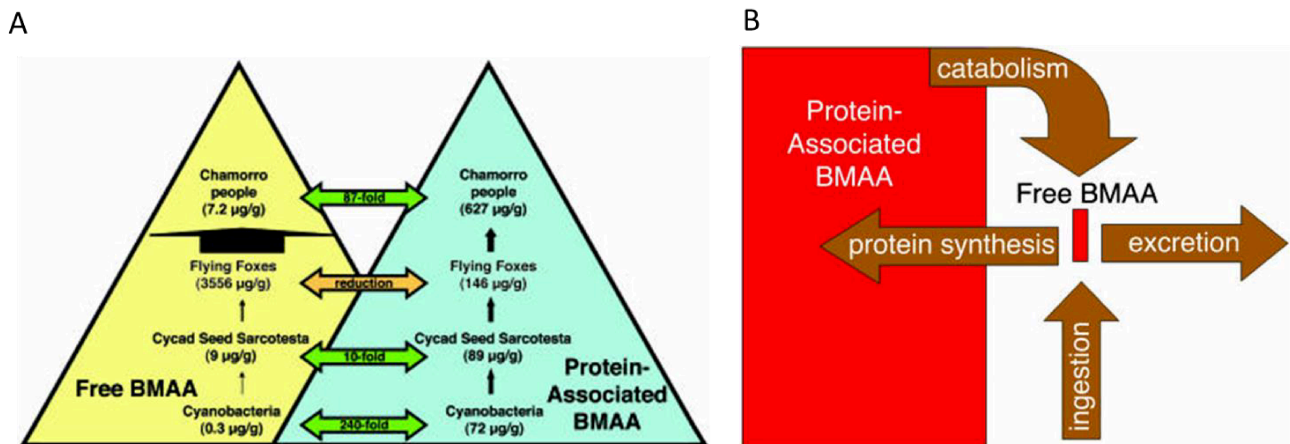
#### a. Epidemiological studies and toxicological value

In 1967, BMAA was discovered on the island of Guam. Special interest was given to this small island in the western Pacific because an abnormally high number of cases of ALS/PDC, a particular form of neurodegenerative disease, were recorded. In fact, the incidence rate was 100 times higher than the international average and implicated in 8-10% of deaths on the island (Banack et al., 2010). Epidemiological data suggested that an environmental toxin could be responsible for this alarming increase in ALS/PDC cases (Garruto et al., 1980). This led to an examination of the food consumed by the local population. Indeed, the Chamorros consume large amounts of flour made from the seeds of cycads, an endemic plant of the island, where BMAA was discovered (Vega, 1967). *In vivo* studies confirmed the neurotoxicity of BMAA, but the concentrations used exceeded those to which the Chamorros could be exposed. In the early 2000s, previous work by Cox and his team observed biomagnification of BMAA at different trophic levels of the Guam ecosystem, as well as other potential sources of contamination for the Chamorros (Banack & Cox, 2003). Interestingly, BMAA would be either present in free form or bound to proteins in organisms (Murch et al., 2004a).

The favored hypothesis is that of biomagnification. Indeed, BMAA would be biomagnified, exposing consumers to high concentrations, particularly through the consumption of bats, a food frequently consumed by the local population. In humans, a neurotoxic stock of BMAA bound to proteins would be present in the brain. When proteins are degraded, BMAA is slowly



released into the brain, causing chronic neurological damage (**Figure 21**). This process would explain the latency period observed before the onset of the first symptoms of the disease. The other hypothesis supported by researchers is that of bioaccumulation of BMAA by its incorporation into proteins.



**Figure 21** - The bioaccumulation of BMAA on Guam Island. Biomagnification process of BMAA in its free and bound forms (A). Schematic representation of the endogenous neurotoxic reservoir (Murch et al., 2004b)

Over the last decade, there has been a decrease in the incidence of ALS-PDC on the island of Guam, partly due to the eradication of bats and changes in the eating habits of the Chamorros (Plato et al., 2003). These observations support Cox et al.'s hypothesis regarding the involvement of bats in ALS-PDC on this island (Banack et al., 2010, 2015).

Since then, several other ecosystems such as Florida (Brand et al., 2010), the Baltic Sea (Jonasson et al., 2010), and around Lake Tau in France (Masseret et al., 2013a) have reported bioaccumulation of BMAA, thus confirming Cox and his team's observations that BMAA is widely spread. Studies have also suggested a possible link, although not proven, between the consumption of food containing BMAA and the formation of ALS clusters (Field et al., 2013; Masseret et al., 2013a). In France, the BMAALS program, which brings together members of the "coordinated methodology network for investigating spatio-temporal clusters of ALS," is an ambitious multidisciplinary project aimed at studying the link between BMAA and ALS (Delzor et al., 2014).

Post-mortem studies have shown the presence of BMAA in the brains of patients with ALS/PDC and Alzheimer's (**Table 5**). These studies thus establish a link between the presence of the

neurotoxin and diseases related to Tau protein (tauopathies). However, BMAA has not been detected in patients who died of Huntington's disease or in control patients without any neurological pathology (Pablo et al., 2009a).

Samples	Substance of interest	Measurements	Pathologies	Detection	References
Brain	BMAA	83% ALS/PDC Chamorro patients 3-10 µg/g (free form) 100% ALS/PDC Chamorro patients 149-1190 µg/g (bound form) Asymptomatic patients 4.8-82 µg/g	ALS/PDC AD	LC-MS	(Murch et al., 2004b)
Frontal Cortex	Tau Protein	Increase level of phosphorylated Tau	8 PD		(Arif et al., 2014)
Brain	BMAA	ALS patients: 256-331 µg/g of BMAA AD patients: 10-228 µg/g of BMAA HD patients: no detectable	13 ALS 12 AD 8 HD	HPLC LC/MS-MS	(Pablo et al., 2009b)
Brain	BMAA	ALS patients: 627 µg/g of BMAA Asymptomatic Chamorro patients: 82 µg/g of BMAA	ALS/PDC	HPLC	(Banack & Murch, 2009)
Cerebrospinal fluid	BMAA	BMAA on CNS – no relationship between AL and BMAA exposure	12 ALS	UHPLC/MS-MS	(Berntzon et al., 2015)
Brain	BMAA	No free or bound BMAA found	20 AD	UHPLC/MS-MS-DC	(Meneely et al., 2016)
Hair	BMAA	Positive correlation between BMAA on hair sample and Shellfish consumption	None	UHPLC/MS-MS	(Downing et al., 2018)

**Table 5** – Detection of the cyanotoxin BMAA on human sample

Increasingly, epidemiological studies focusing on clusters of ALS are trying to investigate not only the lifestyle of affected patients but also the type of exposure (such as maternal exposure) when possible.

## b. *Experimental studies*

Since the discovery of BMAA in the 1960s, numerous *in vivo* studies have been conducted to determine whether BMAA could be a potential etiological factor for neurodegenerative diseases such as ALS. Most of these studies investigate BMAA in acute toxicity mode, meaning that individuals were exposed only once, at a high dose. Acute toxicity studies are distinguished from chronic and sub-chronic studies based on their exposure duration, which may vary according to the species and their lifespan. According to INERIS, acute exposure in humans corresponds to exposure from a few seconds to a few days, sub-chronic exposure to an exposure from a few days to a few months, and chronic exposure from few years to a lifetime. *In vivo* toxicity studies have been summarized in **Table 6**.

**Table 1***In vivo* experiments investigating BMAA toxicity in rats and mice.

Animal model/age	Tested substance	Tested dose-s	Treatment	Observed effects
Rats, young	L-BMAA	$6 \times 10^{-3}$ - $14 \times 10^{-3}$ mmoles/g (0.71–1.7 mg/g)	i.p./single dose	Behavioral dysfunctions
Rats, n.a	L-DAB.HCl	$4,4 \times 10^{-3}$ mmoles/g ( $678 \times 10^{-3}$ mg/g)	i.p./30 h	Motor dysfunctions and hyperactivity.
Rats, n.a	L and D-DAB.HCl	$3.4 \times 10^{-3}$ - $6,2 \times 10^{-3}$ mM (0.5–1 mg/L)	i.p. intracisternal injection	Mortality
Rats, young	L-BMAA.HCl	$6 \times 10^{-3}$ - $14 \times 10^{-3}$ mmoles/g ( $39 \times 10^{-6}$ - $91 \times 10^{-6}$ mg/g)	i.p./single dose	Motor dysfunctions
	D,L-BMAA.HCl	0.35–4 mg/g ( $2.3 \times 10^{-3}$ - $26 \times 10^{-3}$ mmoles/g)		
Rats, adult	L-BMAA.HCl	0.5 mg/day ( $3.2 \times 10^{-3}$ mmoles/day)	i.c.v./up to 60 days	Motor dysfunctions
Rats, n.a	L-BMAA.HCl	0.5 mg daily ( $3.2 \times 10^{-3}$ mmoles/day)	i.c.v./10 days	Motor dysfunctions
Rats, PND9	BMAA <sup>a</sup>	$200 \times 10^{-3}$ - $600 \times 10^{-3}$ mg/g ( $1,7 \times 10^{-3}$ - $5,1 \times 10^{-3}$ mmoles/g)	s.c. (20 µL/g)/2 days	Motor dysfunctions and hyperactivity.
Rats, PND9–10	L-BMAA.HCl	$50 \times 10^{-3}$ - $200 \times 10^{-3}$ mg/g ( $0,3 \times 10^{-3}$ - $1,3 \times 10^{-3}$ mmoles/g)	s.c./2 days	Cognitive deficits, neuronal death.
Rats, PND9–10	L-BMAA.HCl	$50 \times 10^{-3}$ , $200 \times 10^{-3}$ , $600 \times 10^{-3}$ mg/g ( $0,4 \times 10^{-3}$ , $1,7 \times 10^{-3}$ , $5,1 \times 10^{-3}$ mmoles/g)	s.c./2 days	None.
Rats, pups	BMAA.HCl	$460 \times 10^{-3}$ mg/g ( $3 \times 10^{-3}$ mmoles/g)	s.c. (20 µL/g)/2 days	Selective uptake in hippocampus, cell loss, mineralization, astrogliosis, learning and memory impairment in adulthood, protein aggregation. Neuronal death.
Rats, PND21	L-BMAA.HCl	$300 \times 10^{-3}$ mg/g ( $2,5 \times 10^{-3}$ mmoles/g)	i.p./5 days	Neuronal death.
Rats, adult	L-BMAA <sup>a</sup>	$300 \times 10^{-3}$ mg/g ( $2,5 \times 10^{-3}$ mmoles/g)	i.v./2 to 8 weeks	Astrogliosis, neuronal death
Rat pups	L-BMAA <sup>a</sup>	$400 \times 10^{-3}$ mg/g	s.c./single dose at PND 3 to 10	B-amyloid deposition, neurofibrillary tangles, microgliosis, neuronal death
Rat pups	L-BMAA.HCl	$400 \times 10^{-3}$ mg/g ( $3,4 \times 10^{-3}$ mmoles/g)	s.c./single dose at PND3 to 10	Motor dysfunctions and cognitive deficits
Rat pups, PND3	BMAA <sup>a</sup>	$50 \times 10^{-3}$ - $600 \times 10^{-3}$ mg/g ( $0,4 \times 10^{-3}$ - $5 \times 10^{-3}$ mmoles/g)	s.c./single dose	Motor dysfunctions, β-amyloid deposition, neuronal death
Rats, PND5	BMAA.HCl	$400 \times 10^{-3}$ mg/g ( $2,5 \times 10^{-3}$ mmoles/g)	s.c./single dose	Motor dysfunction and neuronal loss
Mice, neonatal	BMAA <sup>a</sup>	1 mg ( $8,5 \times 10^{-3}$ mmoles)	i.c.v./2–2.5 min	Hyperexcitable state, motor dysfunctions
Mice, neonatal	BOAA <sup>a</sup>	0.05 mg ( $284 \times 10^{-6}$ mmoles)	i.c.v./2–2.5 min	Motor dysfunctions
Mice, 2 months	D,L-BMAA.HCl	$500 \times 10^{-3}$ mg/g- $1000 \times 10^{-3}$ mg/g ( $3,2 \times 10^{-3}$ - $6,4 \times 10^{-3}$ mmoles/g)	p.o./11 weeks, daily	None
Mice, n.a	DL-BMAA.HCl	$1 \times 10^{-3}$ - $10 \times 10^{-3}$ mmoles (0.15–1.5 mg)	i.c.v./single dose	Motor dysfunctions
	L-BMAA.HCl	$0,5 \times 10^{-3}$ - $1,5 \times 10^{-3}$ mmoles ( $77 \times 10^{-3}$ -0.2 mg)		
Mice, 6 months	BMAA.HCl	$28 \times 10^{-3}$ mg/g (0.2 mmoles)	p.o./30 days	None
Mice, 12–14 weeks	BMAA <sup>a</sup>	100 mM ( $11,8 \times 10^{-3}$ mg/L)	Intracranial injection	Neuronal death

This table focuses on *in vivo* studies testing BMAA toxicity in mice and rats. As for all tables, a homogenous annotation system was applied. All te expressed in mg/L and mM.

n.a.: not available.

BMAA: β-N-methyl-amino-L-alanine; BMAA.HCl: β-N-methyl-amino-L-alanine hydrochloride; DAB: 2,4-diaminobutyric acid; DAB.HCl: 2,4-diaminobutrochloride; i.v.: intravenous; BOAA: β-N-oxalylamino-L-alanine; i.c.v.: intracerebroventricular; s.c.: subcutaneous; PND: postnatal days; i.p.: intraperito

**Table 6** – Summary of *in vivo* studies on BMAA exposure (Courtier et al., 2022)

The main observed effects are motor dysfunctions, cognitive deficits such as impaired learning and memory, and hyperactivity. Motor dysfunction is one of the main signs of ALS. These motor

disturbances have been observed in rats, mice, and macaques (**Table 6**). Whenever possible, the age of the organisms studied was specified. Indeed, several studies cited here have demonstrated the ability of BMAA to impact the brain functions of adults after embryonic or neonatal exposure, suggesting it as a developmental neurotoxin. In 2009, Karlsson and his team demonstrated that BMAA can be transferred, from the mother to the brains of fetuses and neonates in mice and rats, causing impaired locomotor abilities and hyperactivity, thus suggesting a risk during pregnancy (Karlsson et al., 2009a). Additionally, it has been demonstrated in rats that the toxin is present in milk, and therefore transmitted during offspring nursing (Andersson et al., 2016). It has also been shown that neonatal exposure can lead to changes in ATP and amino acid metabolism, affecting brain function in adulthood (Engskog et al., 2013).

### c. *Neuro-molecular mechanism in vertebrate*

BMAA is a non-canonical amino acid produced by various taxa of phytoplankton, including freshwater cyanobacterial strains, marine diatoms, and dinoflagellates. As previously mentioned, this toxin is thought to be involved in the etiology of sporadic forms of slow-developing neurodegenerative pathologies such as ALS. However, the epidemiological link between human exposure to BMAA and the development of such pathologies remains to be precisely identified. Recent *in vivo* studies have highlighted the impact of BMAA exposure on specific areas of the central nervous system (Delcourt et al., 2018; Pierozan, Piras, et al., 2020; Pierozan & Karlsson, 2019; Scott & Downing, 2019). There are several hypotheses explaining the mechanisms through which BMAA should act on the central nervous system (CNS) :

#### - **BMAA induces excitotoxicity through glutamate receptors**

BMAA was first studied at relatively high concentrations (1 mM to 3 mM) on primary cultures of mouse cortical neurons (Weiss & Choi, 1988a), where the molecule induces neurotoxic and neuroexcitatory effects if physiological concentrations of bicarbonate ions (10 mM) are added to the extracellular medium. The interaction of bicarbonate ion with the toxin provides a carboxyl group necessary for interaction with mGluRs (Weiss & Choi, 1988a). This interaction has also been identified in other *in vitro* studies, including non-neuronal cells such as glial cells (Chiu et al., 2012). Thus, the BMAA/HCO<sub>3</sub><sup>-</sup> interaction is likely to form a molecule structurally related to glutamate, acting as an agonist of its receptors. Several studies

conducted by Pierozan and colleagues support the involvement of glutamatergic mechanisms in BMAA toxicity (Pierozan, Cattani, et al., 2020; Pierozan et al., 2018). Indeed, NMDA receptor antagonist (MK-801) and metabotropic glutamate receptor antagonist (MCPG) protect against BMAA-induced alterations (Pierozan, Cattani, et al., 2020). Interestingly, in primary culture of neurons, the protective effect of CNQX, a non-NMDA ionotropic receptor antagonist, is not observed. This suggests the importance of interactions between neuronal and glial cell populations. This also highlights the involvement of glial AMPA receptors in BMAA-induced toxicity (Pierozan, Piras, et al., 2020).

Considering the increasing number of epidemiological, clinical and experimental evidence strengthening the link between exposure to environmental toxicants like the toxin BMAA or herbicides like GLY or GLA and critic neurological disturbance, it is of paramount of importance to implement experimental studies considering “multiple exposure phenomenon”. To go further, exposome and "multiple-hit hypothesis" notions will be discussed in order to be closer to actual exposure conditions.

### III. Actual exposure condition

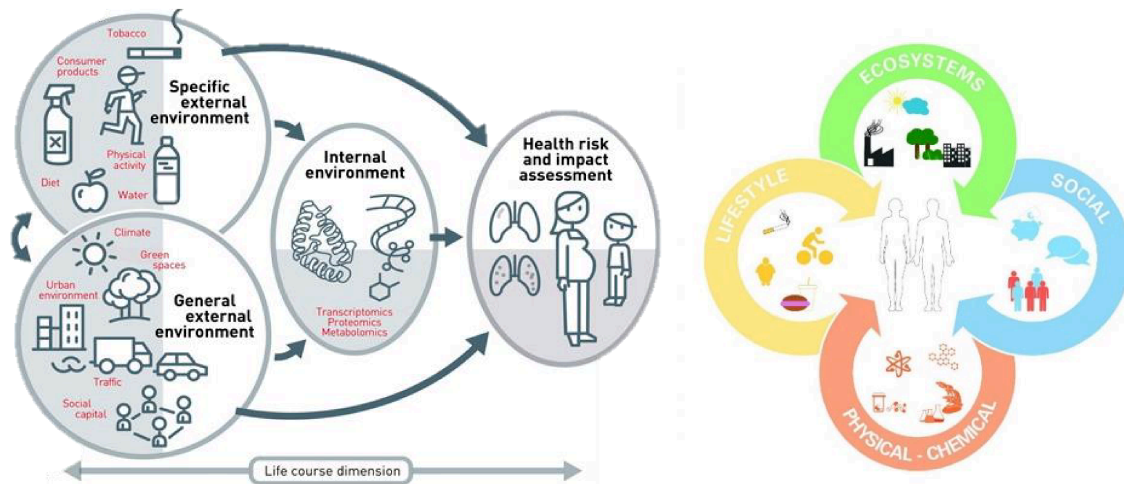
#### 1. Exposome and “Multiple-Hit” Hypothesis”

During their lifetime, each individual is exposed to a variety of factors in an environment that is unique to them. The concept of exposome was introduced in 2005 by the British cancer epidemiologist Christopher Wild. This concept emerged from the necessity to gain a comprehensive understanding of the health implications resulting from the cumulative exposures experienced by individuals over their lifespan, from conception to death, including in utero development, complementing the effect of the genome. Thus, the exposome has been defined as encompassing all factors including (a) physical and chemical factors (radiation, drugs, food contaminants, pesticides ...) (b) psychosocial factors (stress, parental care, education ...) (c) lifestyle factors (sleep, exercise, smoking...) (d) ecosystems and climate (green areas, population density) and (e) alimentation during early life (**Figure 22**).

The evaluation of the exposome necessitates the consideration of an extensive array of variables that have a substantial impact on health, potentially resulting in the development of diseases or the exacerbation of existing conditions. Thus, the challenges of research today are to characterize the different facets of the exposome, measure its effect on the occurrence of

human diseases, including cancers, neurodegenerative diseases, or endocrine-related diseases, and study the possible interactions between the components of the exposome and various biological parameters.

Human health depends on two major components, connected to each other: the genome linked to our genetic makeup and the exposome linked to the environment in which we live or have lived.



**Figure 22** – Schematization of the exposome paradigm defining the three overlapping domains: general external environment, specific external environment and internal environment (Barcelona Institute for Global Health)

The multiple hit hypothesis proposes that complex diseases, such as cancer or neurodegenerative diseases, develop as a result of multiple genetic and environmental factors interacting with each other over time. According to this hypothesis, a single genetic or environmental factor is not enough to cause the disease. Instead, the disease develops only when a combination of multiple factors reaches a certain threshold, leading to a cascade of events that eventually result in the disease. In this hypothesis, each individual hit may be a small effect, but together they create a "perfect storm" that leads to the disease. These hits may occur at different times in a person's life, from before birth to old age, and may involve various factors such as lifestyle choices, environmental exposures, infections, and genetic mutations.

The multiple hit hypothesis suggests that disease prevention and treatment may be more effective if they target multiple factors rather than focusing on a single factor. By identifying and modifying multiple risk factors, it may be possible to reduce the overall burden of disease and improve health outcomes.

## 2. Cocktail effect

The term "cocktail effect" is used to describe the effects that exposure to multiple chemicals simultaneously, even at low doses, can have on human health. The main idea behind the cocktail effect is that if we are exposed to several different chemicals at the same time, it can theoretically enhance the harmful effects of each substance or produce unexpected effects. Currently, scientific knowledge about cocktail effects is relatively limited, but it is the subject of increasingly frequent research. The cocktail effect has been demonstrated in several situations concerning animals, as well as in humans in the case of certain medications. Research on this topic is progressing steadily.

The issue of the cocktail effect is particularly important when it comes to pesticides. Today, most national and international health agencies generally consider that "normal" human exposure to pesticides (i.e., respecting health exposure thresholds) has little impact on health. Indeed, if precautions are taken, the doses to which consumers are exposed through food or the environment are very low. At these doses, scientific experimentation has generally demonstrated an absence of observable harmfulness for the different substances individually, with the exception of controversial pesticides whose use is gradually being regulated.

However, we still have relatively little understanding of the effect that different pesticides can have when we are exposed to them at the same time (including at low doses), i.e., the pesticide cocktail effect. In 2018, a study published in the journal *Environmental Health Perspective* had some initial results on the cocktail effects of pesticides on mice. Researchers exposed mice to low doses of a cocktail of six pesticides, through dilution of the pesticides in a solvent integrated into their diet. These mice developed metabolic syndromes (weight gain, diabetes) and certain specific pathologies. This is a first indication of a possible cocktail effect of pesticides (Lukowicz et al., 2018).

In general, in public health, the toxicity or danger of a substance is evaluated in isolation. Most often, through *in vitro* and *in vivo* tests, cells or organisms are exposed to different doses of a substance and its physiological or biological effects are observed. This allows us to know the toxicity of a substance, its toxic dose, and make recommendations on doses not to exceed.

The problem is that this methodology does not allow us to know the interactions between several substances, what the effects are when exposed to several substances at the



same time. However, living organisms and human beings are constantly exposed to multiple different substances: medications, pesticides, endocrine disruptors, pollutants, contaminants, volatile organic compounds... Theoretically, it is possible that by being exposed to several substances at the same time, some unexpected effects may be observable. Some substances could theoretically be harmless to humans when taken individually, but become toxic when mixed together.

To understand all possible effects of all these substances, it would be necessary to be able to observe their simultaneous effects on an organism. Experimentally, this is quite difficult because to obtain a scientifically reliable result, it is generally necessary to be able to isolate the factors to compare them to a control situation. Therefore, it is difficult to evaluate the cocktail effect, as we generally cannot "isolate" the exposed populations and compare them to "non-exposed" populations. In addition, the peculiarity of the cocktail effect is that it can occur during low-dose exposures, which are difficult to measure precisely. This is why there is currently controversy over the effects of the different chemical and organic substances to which humans are exposed. However, we now have good reasons to believe that simultaneous exposure to numerous chemical substances or pollutants has a significant impact on a few health indicators: immune system, cancer prevalence, obesity, etc. For example, a study have shown that à combined exposure to an herbicide and a pesticide resulted in potentiated effects that appear to target nigrostriatal dopamine systems, suggesting that it is important to consider the combination of environmental risk factors (Thiruchelvam et al., 2000a).

# Main goals and work hypothesis

Over the past few decades, there has been a growing interest in the involvement of environmental factors in the etiology of neurodevelopmental and/or neurodegenerative pathologies. Although research targeting the perinatal period is common in the literature, particularly those concerning the effects of maternal inflammation using the Maternal Immune Activation (MIA) model, few incorporate the notion of low dose. Indeed, the observed effects are often linked to the use of particularly high doses, not necessarily representative of those to which we are daily exposed. The few studies using relatively low doses do not show clear and lasting effects in offspring. In addition, research on these pathologies has focused mainly on exposure to a single factor (pesticides, cyanotoxin, or maternal inflammation), overlooking the notion of the exposome and multiple exposures, which are of paramount importance in the emergence of such disorders.

This thesis aims to approach exposure conditions as close as possible to reality. Therefore, as it has never been evaluated to our knowledge, we hypothesized that a combined exposure to Glyphosate, Glufosinate ammonium, and BMAA, associated or not with inflammatory sensitization, could act in synergy and induce deleterious neurobiological effects. Thus, in an attempt to best address this hypothesis, the main objectives of this thesis were, first, to determine the link between exposure to environmental agents and the late development of neurological disorders. In parallel, the other main objective was to develop an experimental design, approaching "real exposure conditions" by placing our study in an exposome context. This consisted of a concomitant chronic and low-dose exposure to different environmental factors, associated or not with a low inflammatory episode.

In order to evaluate this synergistic effect, various *in vivo* protocols were implemented. In this context, the first *in vivo* protocol consisted in knowing if an inflammatory episode will sensitize a post-natal exposure to low dose of toxicants; to explore the "Multiple-Hit hypothesis". The second one, focused on the evaluation of the "cocktail effect" consisted on a maternal exposure to low doses of BMAA, GLY or GLA either separately or as a mixture.

Following this, a longitudinal behavioral assessment has been conducted in offspring at different time-points, from post-natal period to aging, on different behavioral sphere including

motor behavior. This entire work provides an innovative perspective regarding the involvement of multiple exposures in the toxicological evaluation of long-term effects.

# Part 2: RESULTS

## Paper 1: The herbicides Glyphosate and Glufosinate and the cyanotoxin BMAA induce long-term motor disorders following postnatal exposure: importance of a prior asymptomatic maternal inflammatory sensitization (accepted for publication)

### STUDY BACKGROUND

**Background:** Prenatal Maternal Immune Activation (MIA) and/or perinatal exposure to various xenobiotics have been identified as risk factors for neurological disorders, including neurodegenerative diseases. Epidemiological data suggest an association between early multi-exposures to various insults and neuropathologies. The “Multiple-hit Hypothesis” assumes that prenatal inflammation makes the brain more susceptible to subsequent exposure to several kind of neurotoxins. To explore this hypothesis and its pathological consequences, a behavioral longitudinal procedure was performed after a prenatal sensitization and postnatal exposure to low dose of pollutants.

**Methods:** A maternal exposure to an acute immune challenge (first hit) was induced by an asymptomatic LPS dose (0,008 mg/kg) in mice. This sensitization was followed by exposing the offspring to environmental chemicals (second hit) postnatally, by oral route. The chemicals used were low doses of the cyanotoxin  $\beta$ -N-Methylamino-L-Alanine (BMAA; 50 mg/kg), the herbicide Glufosinate Ammonium (GLA; 0,2 mg/kg) or the pesticide Glyphosate (GLY; 5 mg/kg). After assessing maternal parameters, a longitudinal behavioral was carried out on offspring in order to evaluate motor and emotional abilities at adolescence and adulthood.

**Results:** At first, we showed that the low LPS immune challenge was an asymptomatic MIA. Even though a significant increase in systemic pro-inflammatory cytokines was detected in the dams, no maternal behavioral defects were observed. In addition, as shown by rotarod assays and Openfield device, this prenatal LPS administration alone did not show any behavioral disruption in offspring. Interestingly, our data showed that offspring subjected to both MIA and post-natal BMAA or GLA exposure displayed motor and anxiety behavioral impairments during adolescence and adulthood. However, this synergistic effect was not observed in GLY exposed offspring.

**Conclusion:** These data demonstrated that prenatal and asymptomatic immune sensitization should be a priming effect to subsequent exposure to low doses of pollutants. This double-hits act in synergy to induce motor neurons disease related phenotypes in offspring's. Thus, our data strongly emphasize to consider multiple exposures for developmental neurotoxicity regulatory assessment and pave the way for future studies aiming at deciphering cellular pathways involved in these sensitization processes.

## I. Introduction

Early life events play an important role in health and neurodevelopment of an individual. Throughout life, from the embryonic period until aging, an individual is exposed to a multitude of environmental agents, through various sources such as our diet, pollutants, or microorganisms. During *in-utero* stages, the maternal environment affects the growing fetus. It is well documented that Maternal Immune Activation (MIA), induced by infectious and non-infectious insults, is a serious risk factor of subsequent neuropathologies in offspring's, whereby increasing the level of maternal pro-inflammatory cytokines and interfering with fetal brain development (Estes & McAllister, 2016; Meyer, 2014). MIA has been principally described as an inductor of neuropsychiatric disorders such as neurodevelopmental disorders (Choudhury & Lennox, 2021; Han et al., 2021), but also could be a major factor contributing to neurodegenerative diseases (Nisa et al., 2021; Tartaglione et al., 2015b). However, the majority of maternal infections do not induce permanent disorders in offspring (Estes & McAllister, 2016) suggesting other additional disruptive factors.

Among them, agricultural pesticides exposure during pregnancy and/or lactation is likely to trigger the onset of neuronal adult disorders (Ayeni et al., 2022; Rauh et al., 2012). In fact, experimental studies highlighted the association between several highly used and frequently studied pesticides (e.g Glyphosate) during the perinatal period and disruptive long-term effects in offspring. Thus, Gallegos et al, have shown a delay in sensorimotor reflexes acquisition and an altered locomotor activity and anxiety behaviour in rat offspring following a perinatal exposure to Glyphosate Based Herbicide (GBH) as well as an impairment in recognition memory (Gallegos et al., 2016b, 2018). Further investigations revealed that these behavioral defects were associated with cholinergic/dopaminergic system dysfunction and oxidative stress impairments in offspring (Ait-Bali et al., 2020b; de Oliveira et al., 2022b). Additionally, other work demonstrated that a glyphosate exposure during the prenatal period

leads to motor and cognitive defects in pups, suggested to be linked to a downregulation of Wnt/Ca<sup>2+</sup> pathway (Coullery et al., 2020b). In line with these findings, others herbicides have been also incriminated. Thus, we previously demonstrated that perinatal exposure to low-dose of another well know herbicide, Glufosinate-ammonium (GLA) led to autistic-like behavioural phenotype and neuroblastic migration disturbances (Herzine et al., 2016; Laugeray et al., 2014). More recently, other analysis revealed that perinatal GLA exposure led to an impaired motor coordination functioning in offspring, as well as affecting cortical development, in particular interneuron migration (K.-T. Kim et al., 2020). Dong and collaborators have also demonstrated a reduced locomotor activity and an impaired memory in a mouse model exposed to GLA prenatally (Dong et al., 2020b).

In addition, among environmental toxicants, an increasing number of studies put forward the involvement of non-anthropogenic compounds as potential etiological factor of many neurological disorders. The  $\beta$ -N-Methylamino-L-Alanine (BMAA), a cyanotoxin synthesized by cyanobacteria, is increasingly considered as a potential environmental risk factors for sporadic neurodegenerative outcomes, such as amyotrophic lateral sclerosis (ALS), Alzheimer's Diseases (AD) or Parkinsonism-dementia Complex (PDC) (Caller et al., 2018; Cox et al., 2018; Pablo et al., 2009b). Interestingly, a BMAA post-natal exposure performed in rats induced neurodevelopmental neurotoxicity observed through specific learning impairments (Karlsson et al., 2009b). More recently, we demonstrated, with an extended exposure window, including the prenatal period, short and long-lasting emotional disturbances in offspring (Laugeray et al., 2018).

Despite the great diversity of experimental procedures using wide range of doses associated with different treatment durations and exposure periods, it appears that the perinatal period is a particular sensitive window for the emergence of nervous system disturbances. The pre- and post-natal periods are highly complex and essential time spans for brain development, during which extremely controlled phenomena occur, such as neurogenesis or differentiation and maturation of neuronal precursors (Purves et al., 2012). Modifications to these tightly regulated processes may be responsible for permanent impairments, thereby leading to a wide range of enduring adverse impact later in life. This is the core principle of the Barker' hypothesis better known as the Developmental Origins of Health and Disease (DOHaD) (Sinclair & Singh, 2007). Several hypothesis have been made,

including that environmental agents epigenetically deregulate developmental gene expression disturbing their functions in a long-term manner (Lahiri & Maloney, 2012). Similarly, recent data demonstrated that early exposure to pollutants might disrupt microbiome environment that is able to disturb neurodevelopmental processes (Stiemsma & Michels, 2018).

Thus, these already published studies underlie that each individual could be exposed to a variety of factors that define their exposome. This concept, introduced by Christopher Wild in 2005, was born of the need to better understand the health impact of all exposures (e.g. pollutants) an individual could be exposed to, from conception to death, complementing genome induced effects (Wild, 2005). While multiple exposure may increase the risk of developing major mental illnesses and neurodegenerative disorders, it is possible that there is an epidemiological association between exposure to various insults during pregnancy and neurological disorders. Thus, based on the principle of the “Multiple-hit Hypothesis”, in which it assume that prenatal inflammation makes the brain more susceptible to subsequent exposures to several kinds of neurotoxins. MIA would be a “disease primer” in the brain responsiveness to cumulative lifetime exposure to environmental insults (Kristin L. et al., 2019).

Taken together, our hypothesis is that acute and asymptomatic low dose MIA may act synergistically with low dose pesticides / cyanotoxin exposure to trigger behavioral disruptions in offspring, leading to neurological disease development later in life. To explore the “Multiple-hit Hypothesis”, we investigated the behavioral effects of an early exposure to an acute immune challenge (first hit) induced by an asymptomatic LPS dose (Arsenault et al., 2014), alongside an evaluation of maternal behaviour and the release of pro-inflammatory cytokines. This sensitization was associated with a post-natal exposure to offspring, by oral route, to environmental chemicals (second hit) to low doses of BMAA, GLA or Glyphosate (GLY). A longitudinal behavioral assessment was conducted in offspring in order to evaluate motor and emotional abilities at adolescence and adulthood.

## II. Materials and Methods

### 2.1 Animals, treatment and general procedure

For the experimental procedure, 60 five-week-old female C57Bl/6J mice from Janvier Labs (Le Genest-St-Isle, France) were initially used. Animals were housed and maintained on a 12 h light/dark cycle with ad libitum access to food and drink, at  $21 \pm 1^\circ\text{C}$ , and humidity 50-70% in an EOPS resource facility (UPS44, CNRS, Orléans, France). After three weeks of acclimation, C57Bl/6J mice were bred (two females and one male), in standard Polycarbonate-PC cages (365 x 207 x 140 mm) for mating. Every morning (between 7:30 and 8:00 AM), females were checked for the presence of a vaginal plug to determine if they had been successfully mated during the night, thereby corresponding to gestational day (GD) 0. Pregnant mice were then isolated and randomly divided into two groups, which were administered either LPS 0, 008 mg/kg body weight (L2880-10MG - Lipopolysaccharides from *Escherichia coli* O55:B5, Merck Sigma-Aldrich) or saline solution (NaCl 0, 9%) via intraperitoneal (IP) injection at GD 10, 5 (Hit 1) (Figure 1). From GD0, dams were weighed every two days until delivery. This experiment involved 37 females: 19 saline (Sal) and 18 LPS. In order to verify whether LPS sensitization induces physiological and behavioral changes in dams, body weight was measured every 2 days during the pre-natal period. Maternal behavior was assessed through dams' ability to retrieve their pup at PND3.

One day after birth, the number of male and female offspring was determined and it was decided to choose two male pups at random per litter for incoming adolescent and adult behavioral assessments. The two males were identified via toe clipping methods at PND4. Offspring were exposed *per os*, three times a week, from PND4 to weaning with either BMAA 50 mg/kg bw (B107-50MG, 16012-55-8,  $\geq 97\%$  (NMR), Merck Sigma-Aldrich), GLA 0.2 mg/kg bw (45520-100MG, 77182-82-2, PESTANAL®) or GLY 5 mg/kg bw (45521-250MG, 1071-83-6, PESTANAL®) (Hit 2) (Figure 1). All compounds were prepared in a saline solution of NaCl 0, 9%. Control animals received a comparable dose of the vehicle solution. For practical reasons, we used a 20  $\mu\text{L}$  pipette (P20 with the appropriate tips) to administer the drinkable oral solution directly into pup's mouth. As offspring exposure was carried out orally, litters in saline and LPS groups were separated depending on the type of post-natal exposure (BMAA GLA or GLY) to avoid contaminating other pups with a different treatment, via urine production, licking or suckling. The saline group included: 5 control litters (CTL) (Sal/CTL), 5 litters BMAA (Sal/BMAA),

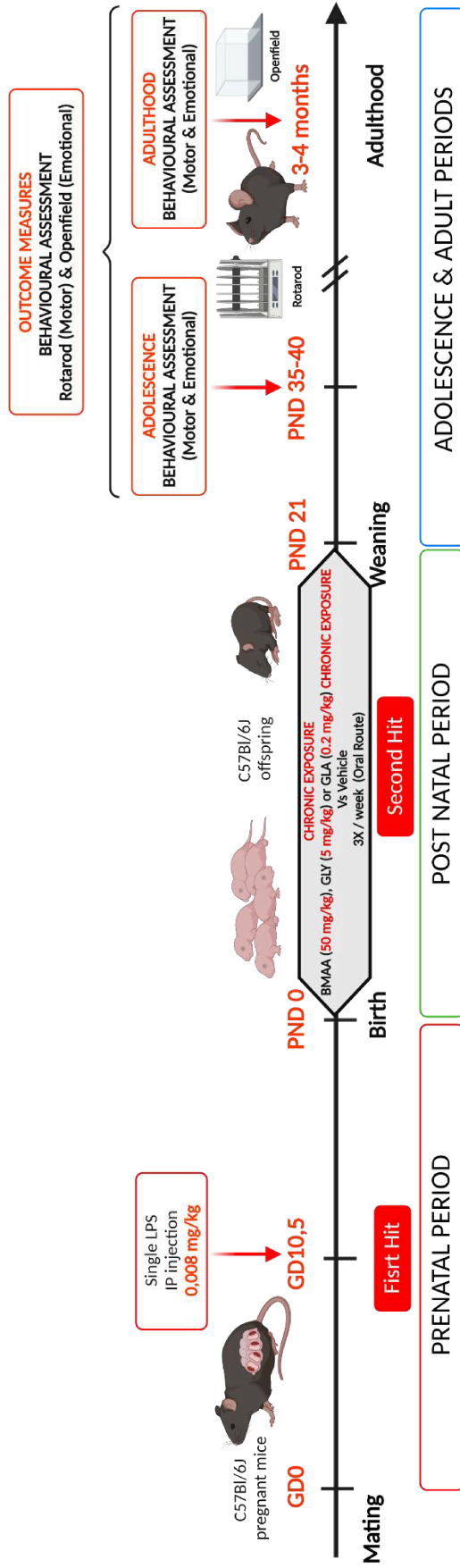


4 litters GLA (Sal/GLA) and 5 litters GLY (Sal/GLY). The LPS group included: 4 litters control (LPS/CTL), 5 litters BMAA (LPS/BMAA), 5 litters GLA (LPS/GLA) and 4 litters GLY (LPS/GLY).

At PND21, offspring were weaned. Labelled males were regrouped per type of pre- and post-natal treatment. Shortly after the end of the treatment, around PND 40, and later during adulthood (3-4 months), a longitudinal behavioral assessment was performed. Two behavioral spheres were assessed: motor and motor coordination behavior using the Rotarod task, and locomotor and emotional behavior with the Openfield test. This experiment involved: n= 10 Sal/CTL, n= 9 Sal/BMAA, n= 8 Sal/GLA, n= 9 Sal/GLY, n= 7 LPS/CTL, n= 9 LPS/BMAA, n= 9 LPS/GLA and n= 7 LPS/GLY.

For cytokine level measurement, 30 female C57Bl/6J wild-type mice were obtained from Janvier Labs (Le Genest St Isle, France) and were bred as described above. Mating and procedure to determine if mice were pregnant was also the same as previously described. Once a vaginal plug had been identified, pregnant mice were weighed every two days until GD 10,5. Twenty-three pregnant mice were obtained and were randomly and equally (as far as possible) assigned to three experimental groups which were administered either LPS 0,008 mg/kg body weight (bw) (L2880-10MG - Lipopolysaccharides from *Escherichia coli* O55:B5, Merck Sigma-Aldrich), LPS 0,1 mg/kg bw or saline solution (NaCl 0,9%) via intraperitoneal (IP) injection at GD 10, 5. Dams were euthanized using an anesthetic overdose of isoflurane (beginning with an isoflurane flow rate of 7% and continuing isoflurane exposure until two minutes after breathing stopped), and mouse peritoneal cells suspension were collected six hours after the LPS or saline IP injection. This experiment involved in total n=23 pregnant females spread into three distinct groups: Saline (Sal; n=7) and LPS Low (0,008 mg/kg bw; n=8) and LPS High (0,1 mg/kg bw; n=8).

All animal care and experimentation were in accordance with the European Communities Council directive (2010/63/EU) and approved by the local Ethics committee (Approval C45-234-6).



**Figure 1 - Experimental design used in the study.** A prenatal immune challenge has been made at Gestational Day 10, 5 in pregnant C57Bl/6J mice with a single intraperitoneal (IP) injection of Lipopolysaccharide at a dose of 0.008mg/kg – First Hit (n= 19 saline (Sal) and n=18 LPS). This maternal sensitization was followed by a postnatal chronic exposure, via oral route, to environmental compounds: BMAA (50 mg/kg), GLY (5 mg/kg) or GLA (0.2 mg/kg) – Second Hit (n= 10 Sal/CTL, n= 9 Sal/BMAA, n= 8 Sal/GLA, n= 9 Sal/GLY, n= 7 LPS/CTL, n= 9 LPS/BMAA, n= 7 LPS/GLY). Outcome measures consisted in a longitudinal behavioral assessment, during adolescence and adulthood. Motor learning and motor coordination behavior were assessed by the Rotarod task and locomotor and emotional behavior with the Openfield test.

## 2.2 Maternal behavior assessment: pup retrieving test

This task was performed to verify whether maternal behavior is modified by a mild inflammatory episode during pregnancy. The retrieval ability was measured by the mother's latency to retrieve the first pup and the total time required to retrieve three pups at PND3. The mother was removed from her home cage for 1 min. Three pups were removed from the nest and then placed in each corner of the cage. The mother was repositioned in her nest, and both the latency to retrieve the first pup and the time to retrieve all three pups were recorded. Retrieval was counted only when the pup was brought fully into the nest, and a score of 180 sec was assigned if the dam failed to retrieve her pups within 3 min.

## 2.3 Adolescence and adult behavior: Rotarod (accelerated mode)

The Rotarod test is used to assess motor coordination and balance in mice. This test was carried out during adolescence (around PND-40) and during adulthood (around 3-4 months). The procedure consisted of 2 distinct phases over 4 consecutive days (Figure 2). One day prior to the test, home cages were placed in the experimental room with the Rotarod device turned on in order to habituate mice to the experimental environment and reduce the effects of stress on their behavior during testing (Day 0).

The first day (Day 1) corresponded to the **Training phase**. Animals were placed on the rotating rod fixed at a speed of 4 rpm until they walked forwards to keep balance. A 60 sec cutoff was defined. Once 60 sec was reached, mice were replaced in their home cage and the apparatus were deep-cleaned with ethanol 70% and water. The procedure was repeated for 3 trials separated by 5 min intersession intervals. Mice had to be capable of staying on the rod for 60 sec before proceeding to testing.

The three following consecutive days (Day 2, 3 and 4) corresponded to the **Testing phase**. Animals were placed on the rod then the apparatus was set to accelerate from 4 to 40 rpm in 5 min. The trial began when the device began accelerating and stopped when the subject fell off the rod. Latency to fall and the rpm associated were the parameters collected for analysis. The procedure was repeated for 4 trials separated by a 3 min intersession interval. After each session, the apparatus was deep-cleaned with ethanol 70% and water.

**Fig. 2**

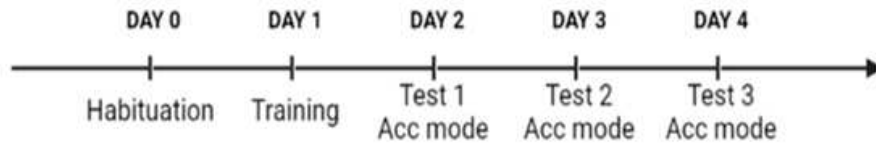


Figure 2 - Rotarod testing procedure

## 2.4 Adolescence and adult behavior: Openfield

The Openfield task was used to assess the ability of mice to deal with unescapable stressful situations. This test was carried out during adolescence (around PND-40) and during adulthood (around 3-4 months). The setup consisted of a 60 cm (length [L]) x 60 cm (width [W]) x 40 cm (height [H]) nontransparent gray Plexiglas arena. The arena was indirectly illuminated with weak white light (30 lx in the center). Each mouse was put in a corner of the open field and allowed to explore the entire apparatus for 10 min. Video tracking software Ethovision (Noldus, Netherland) recorded and automatically analyzed the total distance travelled within the arena and the time spent in two distinct zones: the center and the periphery (sec). The apparatus was deep-cleaned between subjects using a 70% ethanol and water solution.

## 2.5 Cytokine level measurement: ELISA

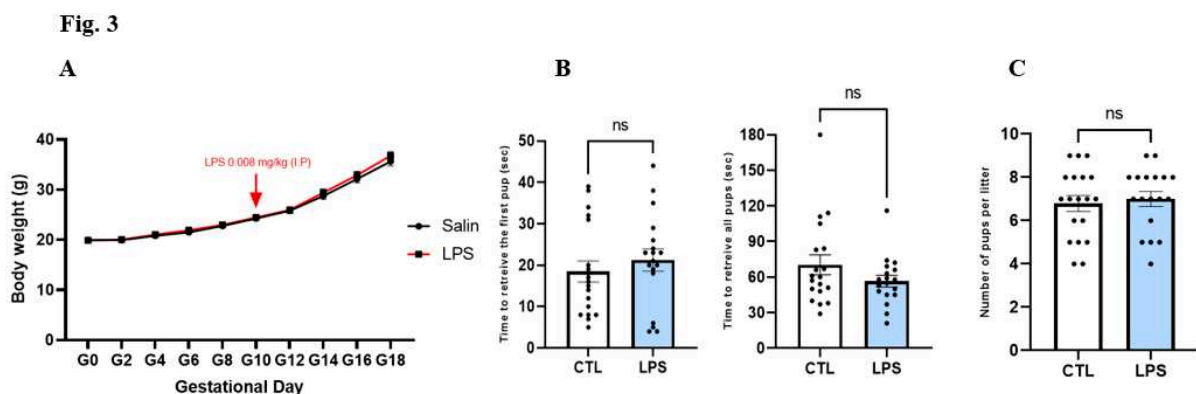
Mouse peritoneal cells suspension were collected 6 hours after LPS intraperitoneal (IP) injection on C57Bl6/J pregnant mice. After mouse euthanasia with an anesthetic overdose (starting with an isoflurane flow rate of 7% and continuing isoflurane exposure until two minutes after breathing stopped), 3 ml of cold PBS was injected into the peritoneal cavity using a 27 G needle. The abdomen was gently massaged and then as much fluid as possible was collected using a 25 G needle attached to a 5 ml syringe. The collected sample was centrifuged at 2000 rpm for 10 min and the supernatant was collected for analysis. The levels of pro-inflammatory cytokine IL-1 $\beta$  and IL-6 (DuoSet<sup>®</sup> ELISA Development System, R&D Systems, Minneapolis, MN, USA) in the peritoneal wash sample were measured using ELISA kits according to the manufacturer's protocol.

## 2.6 Statistical and data analysis

Data analysis was carried out using Graphpad Prism 9 (Graphpad Software, San Diego, CA, USA). Data are presented as mean±SEM for each experimental group. Each point corresponds to an individual. As we are assessing effects on small samples ( $n < 30$ ) and as our data do not follow a normal distribution, we analyzed them, using non-parametric procedures specially adapted for these kind of data. The two main advantages of this statistical procedure compared to a parametric approach are: These methods are not reliant on population assumptions, eliminating the risk of not satisfying prerequisite criteria. Moreover, they demonstrate improved power-efficiency compared to larger sample sizes. Independent two group comparisons were analyzed using the Mann–Whitney test. When more than two groups needed to be consider, the analysis was done using the Kruskal–Wallis “ANOVA-on-ranks” test, and then, when necessary, suitable post-hoc analysis (Dun test) was performed for intra group comparison. When an analysis required repeated measures, a two-way ANOVA with repeated measures was performed. In order to analyze data compared to a hypothetical defined value, a one-sample Wilcoxon test was carried out. P values  $\leq 0.05$  were considered statistically significant.

## III. Results

### 3.1. A low maternal dose of LPS does not alter physiological parameters and maternal behavior

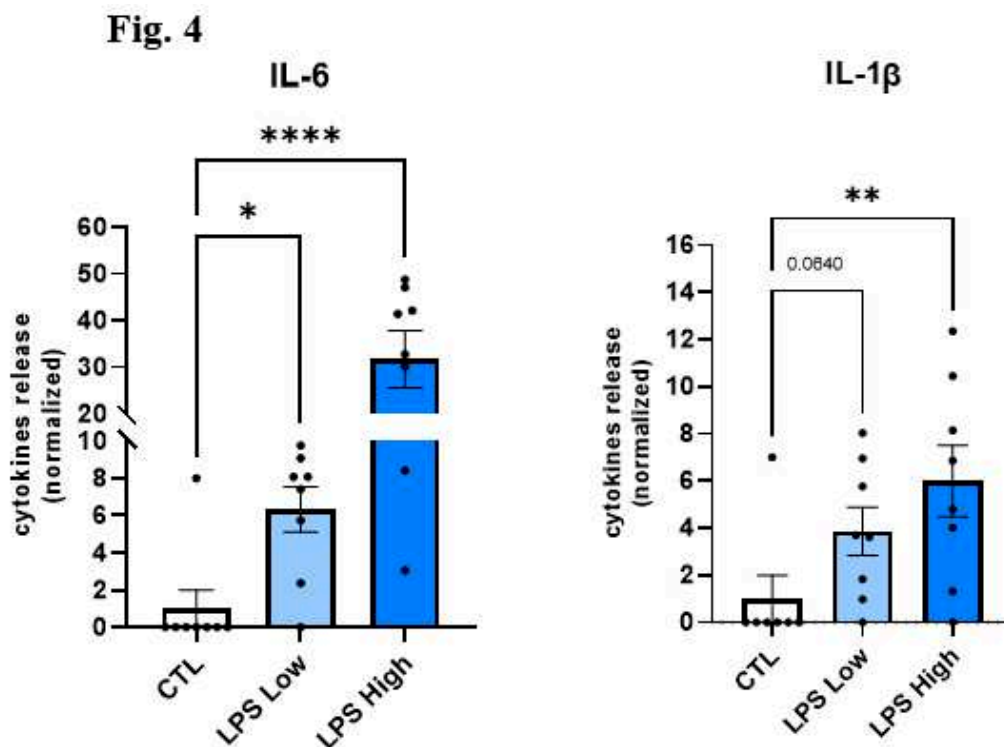


**Figure 3 - A low maternal dose of LPS does not alter physiological parameters or maternal behavior.** Effect of an LPS maternal immune challenge at GD 10,5 (0,008 mg/kg) C57Bl/6 mice on body weight gain during pregnancy (A), on maternal behavior in the pup retrieving task (B) and on the number of pups per litter (C). A low dose LPS did not induce any changes to maternal body weight gain during pregnancy (A). The

latency to return one, followed by all isolated pups was recorded. No significant differences were observed between groups. Data are expressed as mean  $\pm$ SEM. N=19 (CTL), N=18 (LPS). Comparisons were made by a two-way ANOVA (A) and Man-Whitney (B, C).

An immune challenge 0.008 mg/kg dose of LPS did not induce any modification in dams as reflected by the lack of effect on body weight gain during the gestational period (Figure 3A). Additionally, maternal behavior was not shown to be altered by an LPS immune challenge, as time to retrieve the first pup, and mean time to retrieve all pups did not differ between CTL and LPS-sensitized females (Figure 3B). Litter size was not affected either as there was no statistical difference to the mean number of pups per litter between CTL and LPS groups (Figure 3C). In accordance with the literature, the low dose used did not disturb either maternal behavior or body weight.

### 3.2. Low dose of LPS in pregnant female induces a significant increase in pro-inflammatory cytokine levels

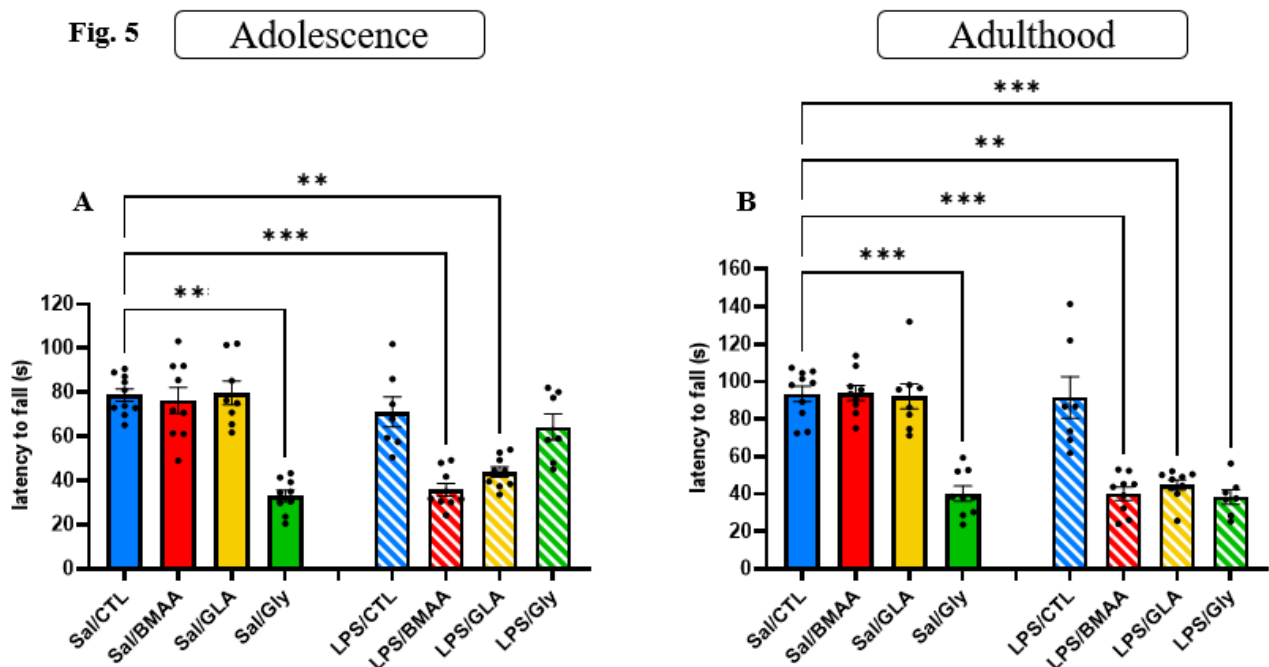


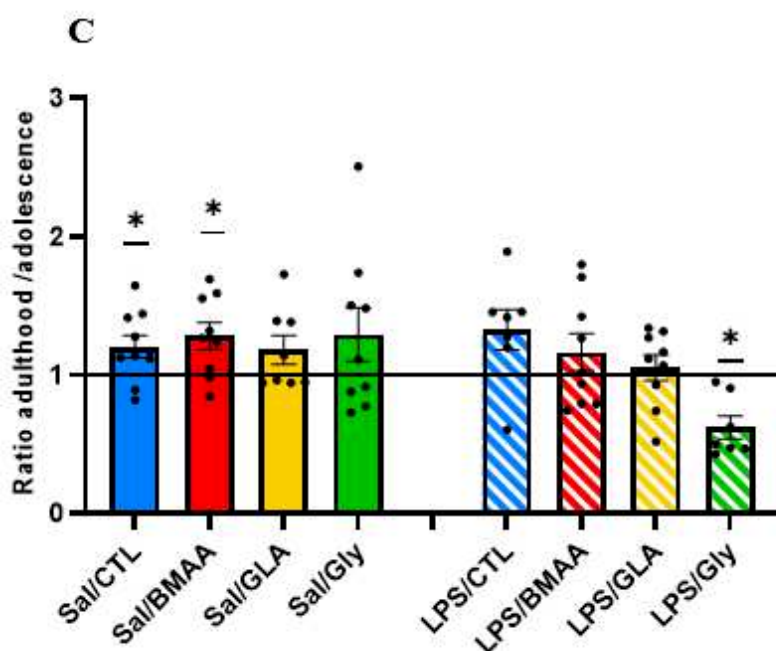
**Figure 4 - Low dose of LPS in pregnant female induces a significant increase in pro-inflammatory cytokine levels.** Effect of the LPS prenatal immune challenge at GD 10,5 on pregnant C57Bl/6 mice on pro-inflammatory cytokine levels in the peritoneal wash. Levels of cytokines IL1- $\beta$  and IL-6 released were measured by ELISA 6 h after an IP injection of LPS at 0,008 mg/kg (Low Dose). For the positive control, pregnant females received an IP injection of LPS at 0,1 mg/kg (High Dose). Data are expressed as mean

±SEM. N=7 (CTL), N=8 (LPS Low Dose), N=8 (LPS High Dose). \*p < 0,05, \*\*p < 0,01, \*\*\*p < 0,001, \*\*\*\*p < 0,0001 (Kruskal Wallis) compared with Sal/CTL group.

We first aimed to determine whether a low dose of LPS prenatal immune challenge triggered the release of pro-inflammatory cytokines, such as IL-6 and IL-1 $\beta$ , which are major mediators of inflammation. ELISA analysis showed that an immune challenge at a low dose of 0,008 mg/kg induced an increase in IL-6 and IL-1 $\beta$ , in the peritoneal wash, 6 hours after the injection (Figure 4). For IL-6, the level was about six times higher than the control, and a trend of about three times higher for IL-1 $\beta$ . As a positive control, females that received a higher dose of LPS (0.1 mg/kg), previously described as inducing a pro-inflammatory response, presented a significant release of both IL-6 and IL-1 $\beta$ , respectively, indicated by an increase of 5 and 30 times more than control (Figure 4). Thus, an LPS prenatal immune challenge at the low dose of 0.008 mg/kg induced a significant release of pro-inflammatory cytokines, even though physiological parameters and behavior were not disturbed.

### 3.3. For motor coordination, an asymptomatic maternal immune challenge sensitizes post-natal response to a low dose of pollutants





**Figure 5 - Asymptomatic maternal immune challenge sensitizes post-natal response to a low dose of pollutants – motor coordination.** Effect of a prenatal immune challenge followed by a postnatal chronic exposure to BMAA (50 mg/kg), GLA (0,2 mg/kg) or GLY (5 mg/kg) on the Rotarod test in adolescence (A) and adulthood (B). The balance and motor coordination of young and adult male mice were assessed in an accelerating mode of the Rotarod test by measuring the latency to fall. Motor learning was represented in a ratio of latency to fall at adulthood and adolescence (C). Data are expressed as mean  $\pm$ SEM. N=10 (Sal/CTL), N=9 (Sal/BMAA), N=8 (Sal/GLA), N=9 (Sal/GLY), N=7 (LPS/CTL), N=9 (LPS/BMAA), N=9, (LPS/GLA), N=7 (LPS/GLY). \*\*p < 0,01, \*\*\*p < 0,001, \*\*\*\*p < 0,0001 (Kruskal Wallis) compared with Sal/CTL group. \*p < 0,05 (One sample Wilcoxon) compared to a hypothetical value of 1.

Balance and motor coordination were assessed in the accelerating mode of the Rotarod test by measuring the latency to fall. During the adolescent period, neither offspring postnatally exposed to either BMAA or GLA, nor those that were prenatally exposed to a low dose of LPS, displayed an impairment to motor coordination (latency to fall was not different to Sal/CTL) (Figure 5A). Data showed that a single asymptomatic prenatal immune challenge was not enough to induce any modification in the latency to fall. However, individuals double-hit with a prenatal immune challenge, followed by chronic exposure to BMAA or GLA fell from the rod significantly faster than the Sal/CTL groups (Figure 5A).

Later, during adulthood, the same profile was observed for this parameter, suggesting a long-term effect (Figure 5B). During adolescence, offspring subjected to a postnatal exposure to GLY



displayed a decrease in the latency to fall from the rotarod, but not after a prenatal immune challenge. In fact, after an asymptomatic prenatal LPS challenge, a resilience was observed in the LPS/GLY group, with no significant differences compared to Sal/CTL (Figure 5A). However, later during adulthood, even though a postnatal exposure to GLY still impaired the balance and motor coordination, a prenatal LPS exposure sensitized a second postnatal offspring exposure to GLY, as the latency to fall became significantly different compared to Sal/ CTL (Figure 5B). In order to evaluate motor learning abilities between adolescence and adulthood, the ratio of latency to fall during adulthood and adolescence was calculated. Only the Sal/CTL and Sal/BMAA groups expressed a motor learning, as the ratio was significantly different to the hypothetical value of 1. Mice from LPS/GLY displayed a ratio significantly lower than 1, illustrating here a drop in motor coordination performance. Nevertheless, all other groups demonstrated insignificant differences between their performances in adolescence and later in adulthood, with a ratio statistically no different to 1 (Figure 5C). Thus, an asymptomatic prenatal immune challenge influenced the post-natal response to a low dose of environmental toxicants in terms of motor coordination performance, and motor learning, with disruption occurring in adolescence and persisting into adulthood

3.4. Asymptomatic maternal sensitization influences the post-natal response to a low dose of pollutants in terms of emotional and locomotor behavior

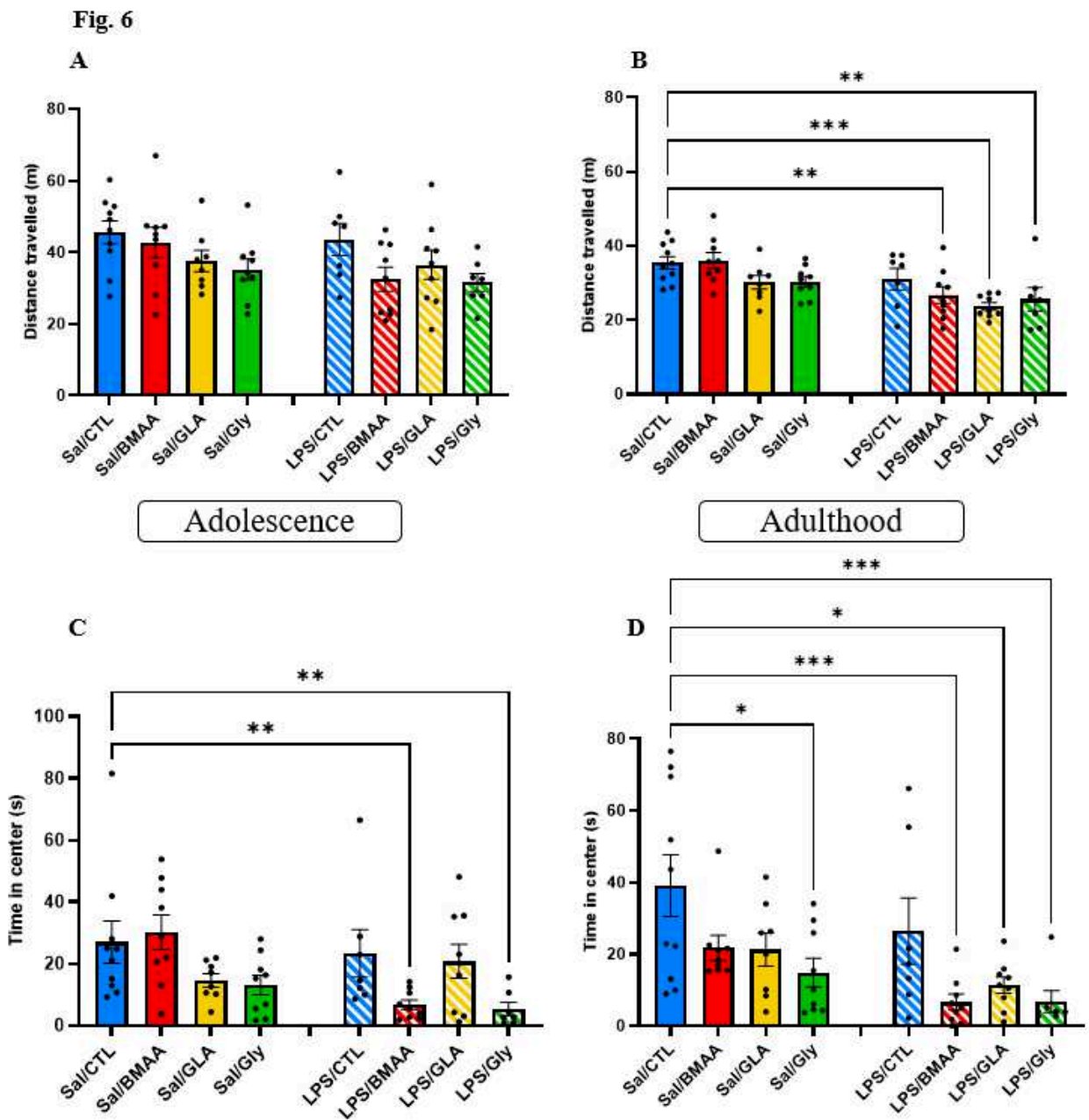


Figure 6 - Asymptomatic maternal sensitization influences post-natal response to a low dose of pollutants - emotional and locomotor behavior. Evaluation of emotional parameters (time spent in the center) and locomotor activity during adolescence (A & C) and adulthood (B & D) in the Openfield. Maternal asymptomatic immune challenge influenced post-natal response to a low dose of environmental toxicants on locomotor activity (A & B) and emotional behavior (C & D). Data are expressed as mean  $\pm$ SEM. N=10 (Sal/CTL), N=9 (Sal/BMAA), N=8 (Sal/GLA), N=9 (Sal/GLY), N=7 (LPS/CTL), N=9 (LPS/BMAA),

N=9, (LPS/GLA), N=7 (LPS/GLY). \*p < 0,05, \*\*p < 0,01, \*\*\*p < 0,001 (Kruskal Wallis) compared with Sal/CTL group.

Anxiety state and locomotor activity were tested using an open-field test. During adolescence, both mice postnatally exposed to either BMAA or GLY, and those that had been prenatally challenged to low dose of LPS, spent a similar amount of time in the centre of the arena, and travelled the same distance compared to Sal/CTL (Figure 6A, C). However, even though the total distance travelled was not impacted, individuals double-hit with a prenatal immune challenge followed by chronic exposure to BMAA or GLY spent significantly less time in the central zone of the open-field. Moreover, a post-natal exposure to GLA, whether combined with an asymptomatic prenatal immune challenge or not, did not induce any significant modification to these parameters (Figure 6A, C). During adulthood, neither offspring exposed to either BMAA, GLA, GLY, nor those subjected to an LPS prenatal maternal immune challenge displayed any modification to general locomotor activity. The total duration spent in the central zone of the open-field was not impacted in these groups except for those exposed postnatally to GLY (Sal/GLY vs Sal/CTL p<0.05\*). Interestingly, groups exposed to a low-dose LPS prenatal immune challenge, followed by a postnatal exposure to BMAA, GLA or GLY, presented a decrease in both the amount of time spent in the center and total distance traveled” (Figure 6B, D). Thus, a maternal asymptomatic immune challenge influenced the post-natal response to a low dose of environmental toxicants in terms of emotional behavior and locomotor activity.

**Table 1**

Adolescence	- LPS			+ LPS			
	BMAA	GLA	GLY	CTL	BMAA	GLA	GLY
Motor coordination	∅	∅	++	∅	++	++	∅
Locomotor activity	∅	∅	∅	∅	∅	∅	∅
Anxiety state	∅	∅	∅	∅	++	∅	++
Adulthood	- LPS			+ LPS			
	BMAA	GLA	GLY	CTL	BMAA	GLA	GLY
Motor coordination	∅	∅	++	∅	++	++	++
Locomotor activity	∅	∅	∅	∅	++	++	++
Anxiety state	∅	∅	++	∅	++	++	++

Table 1. Summary table of post-natal behavioral effect on offspring during adolescent and adult periods after a multiple-hit protocol. ∅ Symbol means that there is no significant effect. ++ means that there are significant modifications.

## IV. Discussion

Converging evidences across epidemiological and experimental studies have identified prenatal Maternal Immune Activation (MIA) and pre- or post-natal exposure to various xenobiotics as a risk factor for neurological disorders, including neurodegenerative diseases (Richardson et al., 2014; Bao et al., 2022). However, the biological processes are complex and the penetrance seems to be incomplete. It suggests that multiple exposure to several insults during the perinatal period should be necessary for the onset of these diseases (Patrick et al., 2019). The present work aimed to explore the concept of “Multiple hit hypothesis”. Firstly, a MIA was induced by an asymptomatic dose of LPS. Next, a second hit consisted by a chronic post-natal exposure to low doses of BMAA, GLA or GLY. Our results suggest that either prenatal immune challenge to LPS or post-natal exposure to xenobiotics BMAA or GLA alone is not sufficient to disturb behavioral performances in adolescence and later in adulthood. However, the double-hit exposure induced significant behavioral changes in offspring in adolescent and adult stages. Our work demonstrates that an asymptomatic maternal sensitization associated with a low-dose exposure to xenobiotics induces neurological disorders. This suggests a molecule dependent synergistic effect in line with the “Multiple-hit Hypothesis”.

Over years, it has been well documented that maternal immune activation, mimed by LPS administration, among others, is a serious risk factor for developing neurological disorders such as neurodevelopmental disorders (Han et al., 2021). Indeed in previous studies, assessing LPS activation during gestation, doses usually needed to range from 0,02 to 0,1 mg/kg in order to induce neurodevelopmental disorders (Hsueh et al., 2017, 2018; Mouihate et al., 2019). However, at these higher doses, there are significant side effects on litter size, due to abortion, foetal death, or behavioral sickness in dams (Kirsten et al., 2010b, 2010a). In the present work, we used a 0,008 mg/kg LPS dose, which is on average 10 to 13 times lower than those usually used in the literature (Boksa, 2010; Arsenault et al., 2014). Our goal was to induce low inflammation with an asymptomatic dose and rather than major disturbances in the pregnant female (Le Belle et al., 2014). In our experimental conditions, we have shown that maternal behavior and physiological parameters such as body weight were not affected by the low LPS immune challenge, even though it induced a significant increase in systemic pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in dams. Based on data, during adolescence, and later in adulthood, mice prenatally exposed to a low dose of LPS had no statistical deviations in general locomotor

activity, anxiety-like, or motor coordination behavior. Interestingly, previous studies discussed the implication that an increase in IL-6 level during pregnancy would have on the cognitive behavior in offspring later in life (Samuelsson et al., 2006). Maternal harsh conditions, including MIA released mediators, may reach the developing foetus by crossing the placenta and then interfere with the ongoing developmental mechanisms of the foetal brain and causing brain disorders in the later life of the affected foetus (Meyer, 2014). In fact, it is clearly established that a maternal immune activating event due to LPS during pregnancy induces the increase of pro-inflammatory cytokines such as IL-1 $\beta$ , leading to short and long term effects in offspring (Rousset et al., 2006). Even though, our data suggest that the circulating cytokines must reach a significant level in order to induce detectable neurodevelopmental defects, silent effect must not be excluded. In human studies, most maternal infections do not lead to neurological disorders in offspring (Estes and McAllister, 2016), suggesting a resilience effect or a protective/adapted response of the organism. In our experimental procedure, the prenatal LPS administration did not show behavioral disruption in offspring, validating our asymptomatic first hit approach.

In regards to our choice of xenobiotic, it has already been established that BMAA, GLA or GLY exposure induced major neurodevelopmental effects. Many epidemiological (Newell et al., 2022; Chang et al., 2023) and preclinical studies have investigated the neurological outcomes of these components. Indeed, it has been shown that a BMAA post-natal exposure of 600 mg/kg leads to motor and cognitive impairments in adulthood (Karlsson et al., 2009b). Similarly, de Oliveira and her team showed that maternal exposure to 50 mg/kg of Glyphosate Based Herbicide (GBH) induced behavioral impairments and oxidative stress in offspring with neurodevelopmental disorders (de Oliveira et al., 2022). In regards to GLA exposure, previous data showed that a maternal exposure of 2 mg/kg reduced locomotor activity and impaired memory in offspring (Dong et al., 2020). In agreement with previous studies, our strategy of using low doses of these molecules showed that mice chronically exposed to either BMAA or GLA during the post-natal period had no detrimental behavioral deviation in adolescence or later during adulthood (Table 1). However, few previous studies explore the effect of a low dose of these kinds of agents. Studies in rodent have shown that a chronic exposure to a low dose of BMAA induce only a mild effect, if any, on behavioral performances both in adult and offspring (Cruz-Aguado et al., 2006; Laugeray et al., 2018). The disparity of these results has

been discussed and can be explained in particular by the low transport of BMAA into adult rodent brain compared to brains of neonatal rats (Karlsson et al., 2009a). Even though BMAA related neurotoxicity has mainly been investigated from the perspective of neurodegeneration, few studies have reported that early exposure to a low dose of BMAA, especially during the neonatal period, may exert subtle neuroanatomical changes leading to behavioral alteration rather than neurodegeneration (Karlsson et al., 2015). In line with these findings, previous work conducted by our team, demonstrated that perinatal exposure to GLA at 0, 2 mg/kg led to neuroblastic migration disturbances but not associated to behavioral impairment (Laugeray et al., 2014; Herzine et al., 2016). Compared to post-natal exposure to BMAA and GLA, mice chronically exposed to GLY during the same period displayed motor coordination deviation from adolescence that persisted later in adulthood. Other studies have already demonstrated that higher doses induce disturbances to locomotor activity and anxiety behaviors in rodents, for both adult and offspring (Hernández-Plata et al., 2015; Gallegos et al., 2016; Baier et al., 2017). In agreement with previous literature, it seems that high-level exposure to GLY may exert a neurological deleterious effect independently of the exposure period. However, to our knowledge, our current study is the first to evaluate offspring behavior after a post-natal exposure to low dose of GLY. Indeed, the GLY dose we assessed here is one-tenth of the current “No Observable Adverse Effect Level” (NOAEL), making the post-natal period particularly sensitive to GLY. These results therefore demonstrated that although these xenobiotics did not induce significant or major effect at these doses, a low dose GLY exposure leads unexpectedly to motor and anxiety behavior impairments.

Our multiple-hit strategy shows that greater alterations to neurodevelopmental trajectories were observed in offspring exposed to both prenatal immune activating event and a chronic post-natal xenobiotics. During adolescence, offspring prenatally subjected to a maternal immune sensitization and exposed chronically to BMAA displayed impairment in motor coordination and an increase of anxious behavior. These behavioral disturbances were still observed later during adulthood, as well as a decrease in general locomotor activity. In line with these results, the LPS/GLA group presented a deficit in motor coordination from adolescence, confirmed later in adulthood during which an increase in anxiety like-behavior was observed as well as a decrease in general locomotion. However, as describe in Table 1, the effects induced by post-natal GLY exposure, were not potentiate by a maternal LPS sensitization (Table 1).

Interestingly, we show that a post-natal chronic exposure to low doses of either BMAA or GLA was not enough to induce behavioral disturbances in offspring. These findings demonstrate that a single risk factor may not be sufficient to cause enduring and long-lasting modifications in behavior. This is in accordance with a previous study conducted by Guma and her co-workers, which showed that mice either prenatally subjected to MIA in early gestation, or postnatally exposed to chronic THC during adolescence did not exhibit persistent behavioral abnormalities later in adulthood (Guma et al., 2022). This *in vivo* analysis revealed a cumulative effect of prenatal MIA and adolescent cannabis exposure on neurodevelopment, suggesting that “multiple hits”, within an individual are required for disease onset. Accordingly, in our experimental design using perinatal pollutant exposure, we also put in light a synergistic effect of two different hits; including prenatal MIA succeeds by post-natal exposure to xenobiotics. Taking together, all results demonstrate that MIA sensitization depends on second hit nature, suggesting that several pathways could be affected and may be investigated in future researches.

Recent clinical and preclinical findings suggest that MIA induces dysregulation of fundamental neurodevelopmental program. MIA therefore affects juvenile and adult brain responsiveness to a cumulative exposure to harmful environmental insult during juvenile period, adulthood and aging (Krstic et al., 2012; Knuesel et al., 2014). These long-term impairments to brain function and behavior therefore often result from early life events. The early life of an individual is composed of multiple critical windows during which developing events occur. The pre- and post-natal periods are essential for brain development, and highly sensitive in term of exposure to any environmental stimuli. Proliferation and differentiation of neuronal precursors, as well as establishment of neuronal circuits lead to the formation of the fundamental structure of the brain. This critical window of brain development, better known as the Brain Growth Spurt (BGS) is also characterized by a significantly high rate of development and proliferation of glial cells, as well as the maturation and invasion of microglia in the spinal cord and the brain (Tay et al., 2017). In rodents, it occurs during the first 3–4 weeks after birth and the corresponding period in Human starts during the last trimester of pregnancy and continues until at least 2 years after birth (Dobbing and Sands, 1979). Thus, disturbances of these tightly regulated processes may negatively affect the establishment of basic neuronal circuits, weakening the fundamental structure of the brain. This could be at the origin of

permanent impairments, leading to a wide range of enduring adverse impact later in life. It thus confers a greater importance to multiple-hit phenomenon, which needs to be investigated further, particularly during these highly sensitive periods of brain development.

We found that exposure to a single risk factor, either prenatal immune sensitization to low dose of LPS or a chronic postnatal exposure to xenobiotics are not sufficient to induce early and long lasting behavioral changes in offspring. In contrast, exposure to both risk factors may induce early and persistent behavioral impairment. Indeed molecular, structural or epigenetic modifications could be at the origin of an excessive reactivity to a further second-hit. It put MIA as a “disease primer” (Meyer, 2014) to make an individual more sensitive to subsequent environmental exposures in triggering disease-related symptoms later in life (Ayhan et al., 2016). While work focusing on interactions between MIA and environmental risk factors steadily increase, animal model outcomes suggest that even subclinical maternal infection can make offspring much more vulnerable to second “hits” (Knuesel et al., 2014). Sub-threshold MIA increases the likelihood of environmental risk factors, such as stress and drug use, to cause neurodevelopmental phenotypes in offspring. It is important to underline that multiple stress exposure is common during developmental period and most of early life stressors are interdependent when it comes to inducing persistent neuroanatomical and behavioral disturbances. In fact, timing of exposure is paramount of importance, as specific phenomena occur at different time points in brain development and induce differential effects. It is paramount of importance to better understand cellular and molecular mechanisms underlying these impairments. In the last decade, an increasing number of studies put forward the importance of the gut-brain axis. In fact, microbiome disruption, induced by several environmental factors, could lead to neurological disorders (Stiemsma and Michels, 2018). It has been demonstrated that GLY and GBH inhibit the Shikimate pathway in plant. Even if this this pathway is not found in animal cells, it is essential to the metabolism of some bacterial species found in human gut microbiota (Mesnage et al., 2021). Previous works sustained that chronic and sub-chronic exposure to GLY or GBH induce an alteration in gut microbiome composition; e.g. decrease levels of some microbes like Lactobacillus, Bacteroides or Firmicutes (Bali et al., 2019). This microbiome GBH dependent imbalance induces an increase of pro-inflammatory cytokine and reactive oxygen species (ROS) production, which have implication on neurodevelopmental impairments (Barnett et al., 2022). In line with this findings, other



studies underline disturbances of Central Nervous System (CNS) development and functioning after a modification of intestinal environment induced by BMAA or GLA exposure (Dong et al., 2020; Esteves et al., 2023). Based on the impact of our three molecules on the microbiota, all these data suggest disruptions of the gut-brain axis in our exposure model. Further studies should be performed to help illuminate the potential connection between the microbiota and consequences of a “multiple-hit” exposure.

## Conclusion

To conclude, our results show that asymptomatic maternal inflammation sensitizes deleterious effects of subsequent low doses xenobiotics exposure on both motor coordination and locomotion. To our knowledge, our work is one of the first using longitudinal design extending from early pregnancy to adulthood to highlight the crucial importance of asymptomatic maternal inflammation in precipitating environmental contaminant-induced motor neuron disease-like symptoms. These findings raise an important public health issue in terms of the evaluation of the toxicological effects of various molecules, requiring further clinical, pre-clinical and epidemiological investigations taking in count multiple-exposures to several insults into account. Therefore, it strongly emphasize to consider multiple exposures for developmental neurotoxicity regulatory assessment and pave the way for future studies aiming at deciphering cellular pathways involved in these sensitization phenomena, leading ultimately to build new adverse outcome pathways.

## Paper 2: Maternal exposure to a pollutant mixture induces long lasting motor coordination impairment and emotional disturbances in offspring's: importance of considering "cocktail effect" (in preparation)

### STUDY BACKGROUND

**Background:** Throughout their life, an individual is exposed to a multitude of social and environmental factors that define their exposome. An increasing number of studies are attempting to incorporate this concept into the understanding of neurological disorders. Epidemiological data suggest an association between early multi-exposures to various insults and neuropathologies. The «cocktail effect» assumes that exposure to multiple chemicals simultaneously, even at low doses, can have even more detrimental effect on human health. To explore this hypothesis and its pathological consequences, a behavioral longitudinal procedure was performed after maternal exposure to low doses of pollutants alone or as a cocktail by intranasal route.

**Methods:** A maternal chronic exposure to environmental chemicals was made by intranasal route from gestational day 4 to delivery. The chemicals used were low doses of the cyanotoxin  $\beta$ -N-Methylamino-L-Alanine (BMAA; 50 mg/kg), the herbicide Glufosinate Ammonium (GLA; 0,2 mg/kg) and the pesticide Glyphosate (GLY; 5 mg/kg), as a cocktail or separately. After assessing maternal parameters, a longitudinal behavioral was carried out on offspring in order to evaluate motor and emotional abilities at post-natal stage, adolescence and adulthood.

## I. Introduction

In recent decades, the number of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson disease (PD) or Amyotrophic Lateral Sclerosis (ALS) has steadily increased. It has long been considered that these pathologies were primarily caused by genetic factors (Bertram & Tanzi, 2005). Nevertheless, more and more studies tend to show that these diseases are the result of a combination of genetic and environmental factors, a concept commonly referred to as the "multiple-hit" hypothesis (Lahiri & Maloney, 2010). This concept suggests that environmental exposures work together to "sensitize" brain tissue, making it more vulnerable to subsequent exposure later in life. Among these environmental factors, different kinds of exposure are considered like exposure to xenobiotics including pesticides or to various toxins (Guma et al., 2022). This concept of multiple effects is reinforced by an increasing number of studies that have shown that early exposure to such agents, especially during the perinatal period, is likely to alter brain development "faith" through neuronal, glial and/or inflammatory processes. Indeed, this period represents a critical window of brain development (Dobbing & Sands, 1979b; Tay et al., 2017). Among other processes, alterations in neural progenitor production, proliferation and neuronal migration are highly suspected to be central in such phenomenon (Yadav et al., 2021). These core principles are the basis for the Baker's hypothesis better known as the Developmental Origins of Health and Diseases (DOHaD) approach (Sinclair & Singh, 2007). According to this concept, the susceptibility to some adult onset-diseases, including neurodegenerative diseases, may originate from in utero and neonatal environmental exposures (Tartaglione et al., 2015b).

Among environmental factors likely to be associated, xenobiotics such as pesticides challenge us as their possible involvement in neurological disorders (Ayeni et al., 2022; Rauh et al., 2012). Based on previous published data including our work, several xenobiotics have been pointed out. It is the case of the  $\beta$ -N-methylamino-L-alanine (L-BMAA), a cyanotoxin produced by more than 95% of all cyanobacterial and microalgae species (Cox et al., 2018). This toxin is increasingly considered as a potential environmental risk factor for sporadic neurodegenerative diseases outcome, such as AD, ALS and amyotrophic lateral sclerosis and parkinsonism-dementia complex (PDC) (Cox et al., 2018; Torbick et al., 2018). L-BMAA represents a global public health concern due to the chronic exposure of humans and other species by recreational waters and seafood consumption (Masseret et al., 2013b; Réveillon et

al., 2015b). Recent findings have shown high levels of BMAA in sharks and dolphins, with neurodegenerative alterations (Hammerschlag et al., 2016; Mondo et al., 2012). Recently, our laboratory demonstrated that a BMAA perinatal exposure induced light long-lasting behavioural disturbances in offspring's (Laugeray et al., 2018). A correlation between high frequency of ALS cases and proximity of lake with cyanobacteria contamination has been reported recently (Torbick et al., 2018). Moreover, ALS and PDC BMAA exposure induced cases have been observed in intensive agriculture areas where many compounds like pesticide are commonly used (Masseret et al., 2013b). Glyphosate (GLY) the most used herbicide worldwide is a systemic, broad-spectrum and post-emergent herbicide (Soares et al., 2021). Gallegos et al, have shown an altered locomotor activity and an anxiety behaviour in rat offspring after a perinatal exposure to Glyphosate Based Herbicide (GBH) as well as an impairment in recognition memory (Gallegos et al., 2016b, 2018). It is known that a perinatal GBH exposure induced oxidative stress impairments in young rats associated with neurodevelopmental disorders (de Oliveira et al., 2022b). Interestingly it has been shown that GLY promote high proliferation of cyanobacteria bloom trough eutrophication phenomenon, making cyanobacteria particularly resistant to this compound (W. Wang et al., 2021). Therefore, the combine use of GLY and Ammonium Glufosinate (GLA), on other well-known herbicides, has been proposed in attempt to counteract the plant resistance to GLY (Ganie & Jhala, 2017b; Samsel & Seneff, 2013b, 2015b). We previously demonstrated that perinatal exposure to low-dose of GLA led to autistic-like behavioral phenotype and neuroblastic migration disturbances (Herzine et al., 2016; Laugeray et al., 2014). More recently, other analysis revealed that perinatal GLA exposure led to an impaired motor coordination functioning in offspring's, but also to an affected cortical development, particularly by affecting interneuron migration (K.-T. Kim et al., 2020). All these data suggest that exposure to a cocktail of these three compounds it is possible and could result in highly deleterious and synergistic effects, which have never been evaluated yet.

Considering the increasing number of epidemiological, clinical and experimental evidence strengthening the link between exposure to environmental toxicants and late onset of neuropathologies, it is of paramount of importance to implement experimental studies that are closer to actual exposure conditions. To explore this "cocktail effect", we investigated the behavioral effects of a chronic maternal exposure to low doses of either BMAA, GLY or GLA

alone or as a cocktail by intranasal route. A longitudinal behavioral assessment has been conducted in male offspring in order to evaluate sensorimotor, cognitive and emotional abilities, during post-natal period, adolescence and adulthood.

## II. Materials and Methods

### 3.1 Animals, treatment and general procedure

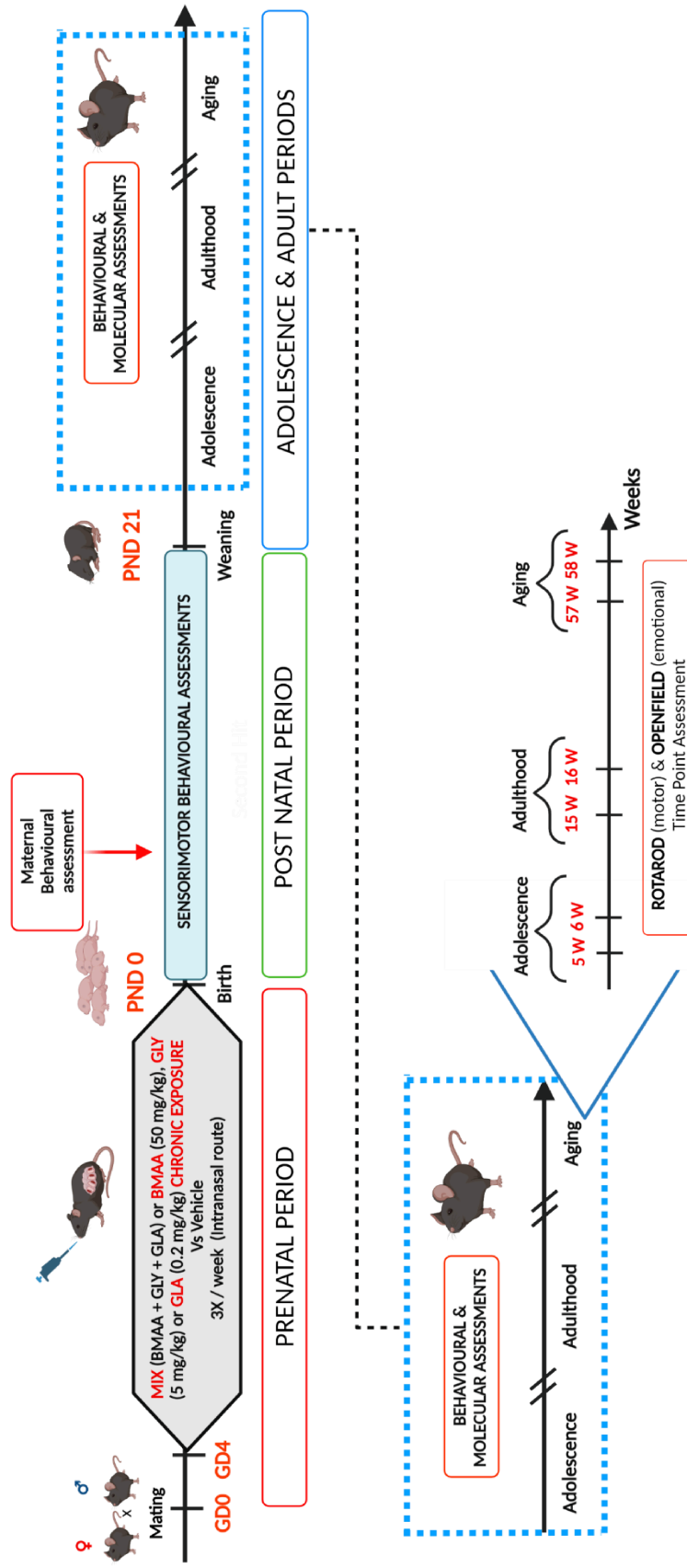
For the experimental procedure, 50 five-week-old female C57Bl/6J mice from Janvier Labs (Le Genest-St-Isle, France) were initially used. Animals were housed and maintained on a 12 h light/dark cycle with ad libitum access to food and drink, at  $21 \pm 1^\circ\text{C}$ , and humidity 50-70% in an EOPS resource facility (UPS44, CNRS, Orléans, France). After three weeks of acclimation, C57Bl/6J mice were bred (two females and one male), in standard Polycarbonate-PC cages (365 x 207 x 140 mm) for mating. Every morning (between 7:30 and 8:00 AM), females were checked for the presence of a vaginal plug to determine if they had been successfully mated during the night, thereby corresponding to gestational day (GD) 0. Pregnant mice were then isolated and randomly divided into two groups, which were administered either a cocktail of toxicants containing BMAA 50 mg/kg bw (B107-50MG, 16012-55-8,  $\geq 97\%$  (NMR), Merck Sigma-Aldrich), GLA 0.2 mg/kg bw (45520-100MG, 77182-82-2, PESTANAL<sup>®</sup>) and GLY 5 mg/kg bw (45521-250MG, 1071-83-6, PESTANAL<sup>®</sup>) or saline solution (NaCl 0, 9%) via intranasal route (IN) from GD4 to delivery (Figure 1). All compounds were prepared in a saline solution of NaCl 0, 9%. Control animals received a comparable dose of the vehicle solution. From GD0, dams were weighed every two days until delivery. This experiment involved 21 females: 10 control (CTL) and 11 MIX. In order to verify whether exposure to a mixtures of toxicants induces physiological and behavioral changes in dams, body weight was measured every 2 days during the pre-natal period. Maternal behavior was assessed through dams' ability to retrieve their pup at PND3 as well as their capacity to build a suitable nest from the last treatment to PN6. A second similar experiment was made to increase the number of offspring for adolescent, adult and aging behavior. This one involved 10 females: 5 control (CTL) and 5 MIX.

One day after birth, the number of male and female offspring was determined and it was decided to choose one male pups at random per litter for incoming adolescent and adult and aging behavioral assessments. One male was identified via toe clipping methods at PND4. All pups were weighed and subjected to a series of pre-weaning tests to check the effects of maternal exposure to the cocktail of toxicants on postnatal development. From PND1 to PND15 communicative skills and sensorimotor development were assessed every day.

At PND21, offspring were weaned. Labelled males were regrouped per type of pre-treatment. Shortly after the end of the treatment, around PND 40, during adulthood (3-4 months), and later during aging (14-15 months), a longitudinal behavioral assessment was performed. Two behavioral spheres were assessed: motor and motor coordination behavior using the Rotarod task, and locomotor and emotional behavior with the Openfield test. This experiment involved: n= 14 CTL, n= 15 MIX (data were pooled from 2 independents protocols).

For the experimental procedure involving a single chemical maternal exposure, 60 female C57Bl/6J wild-type mice were obtained from Janvier Labs (Le Genest St Isle, France) and were bred as described above. Mating and procedure to determine if mice were pregnant was also the same as previously described. Once a vaginal plug had been identified, pregnant mice were then isolated and were administered either BMAA 50 mg/kg bw, GLA 0.2 mg/kg bw or GLY 5 mg/kg bw or saline solution (NaCl 0, 9%) via intranasal route (IN) from GD4 to delivery. This experiment involved in total n=38 pregnant females spread into four distinct groups: Control (CTL; n=10), BMAA (n=9), GLA (n=9) and GLY (n=10). As previously, the number of male and female offspring was determined and it was decided to choose one male pups at random per litter for incoming adolescent and adult and aging behavioral assessments. One male was identified via toe clipping methods at PND4. This experiment involved finally: n= 9 CTL, n= 8 BMAA, n=8 GLA and n=9 GLY.

All animal care and experimentation were in accordance with the European Communities Council directive (2010/63/EU) and approved by the local Ethics committee (Approval C45-234-6).



**Figure 1 - Experimental design used in the study.** A prenatal chronic exposure, via intranasal route, to environmental compounds: BMAA (50 mg/kg), GLY (5 mg/kg) or GLA (0.2 mg/kg), as a cocktail or separately has been made from Gestational Day 4 to delivery in pregnant C57Bl/6J mice. Outcome measures consisted in a longitudinal behavioral assessment, during post natal period and adolescence, adulthood and aging. Motor learning and motor coordination behavior were assessed by the Rotarod task and locomotor and emotional behavior with the Openfield test. Final behavioral cohort: Experiment 1 (MIX exposure) N=14 CTL and N=15 MIX. Experiment 2 (single toxins exposure) N=9 CTL, N=8 BMAA, N=8 GLA, N=9 GLY

### 3.2 Maternal behavior assessment:

#### a. Pup retrieving test

This task was performed to verify whether maternal behavior is modified by an exposure to a cocktail of pollutant during pregnancy. The retrieval ability was measured by the mother's latency to retrieve the first pup and the total time required to retrieve three pups at PND3. The mother was removed from her home cage for 1 min. Three pups were removed from the nest and then placed in each corner of the cage. The mother was repositioned in her nest, and both the latency to retrieve the first pup and the time to retrieve all three pups were recorded. Retrieval was counted only when the pup was brought fully into the nest, and a score of 180 sec was assigned if the dam failed to retrieve her pups within 3 min.

#### b. Nest building

This task was performed to verify whether the ability to build a suitable nest is not impacted by an exposure to a cocktail of pollutant during pregnancy. Pregnant females were maintained on their home-cage to avoid engendering stress just before delivery. Three pressed stack of oval cotton pads were placed on the home-cage and quality of the nest was assessed every day from the last treatment just before delivery until PND6. A score from 1 (worst) to 5 (best) was determinate, based on the procedure described by Deacon (Deacon, 2006).

### 3.3 Post-natal behavior: sensorimotor analysis

In the investigation of early postnatal sensory and motor development, six parameters were monitored:

- **Negative geotaxis:** the pup was placed on its back on a 45° angle slope with its head pointing down the incline (PND1–8). Each day we recorded the percentage of pups that had acquired the reflex.
- **Righting reflex:** when the pup was placed on its back on a flat, hard surface, it had to right itself on all four paws. Two successes in three trials gained a score of 1. A failure was recorded when the pup remained on its back for more than 10 s. For this, a score of 0 was given. Each day, we recorded the percentage of successful pups. A pup was recorded as successful when he got two successes in three trials (from PND1 to PND7).



- **Vertical progression:** the pup was held against a vertical metallic grid (mesh, 6 mm wide). Climbing was scored when the pup passed five stitches (PND4–12).
- **Bar grasping:** the forepaws were placed on a round wooden bar (7 mm diameter). The ability to hang for 10 s using the forepaws was scored (PND8–15).
- **Eyelid opening:** defined as any visible break in the membrane covering the eye. We examined pups from PND10 to PND15 and determined the percentage of pups with at least one eyelid opened).
- **Acoustic startle reflex:** from PND8 to PND14, a small hand-held clicker generated a loud noise and the jerk behavior that immediately followed was scored. The percentage of pups with the reflex was noted.
- **Ultrasonic Vocalizations:** Ultrasonic vocalizations were measured to assess the pups' communicative skills. Vocalizations were recorded from PND1 to PND15. Each animal was tested on each of these days during a 3-min period. After the 3-min recording session, each pup went through all the other pre-weaning tests. Each day we measured the number of calls emitted during the 3-min period by each pup.

### 3.4 Adolescence and adult behavior

#### a. Rotarod (accelerated mode)

The Rotarod test is used to assess motor coordination and balance in mice. This test was carried out during adolescence (around PND-40), during adulthood (around 3-4 months) and later during aging (around 13-14 months). The procedure consisted of 2 distinct phases over 4 consecutive days. One day prior to the test, home cages were placed in the experimental room with the Rotarod device turned on in order to habituate mice to the experimental environment and reduce the effects of stress on their behavior during testing (Day 0).

The first day (Day 1) corresponded to the **Training phase**. Animals were placed on the rotating rod fixed at a speed of 4 rpm until they walked forwards to keep balance. A 60 sec cutoff was defined. Once 60 sec was reached, mice were replaced in their home cage and the apparatus were deep-cleaned with ethanol 70% and water. The procedure was repeated for 3 trials separated by 5 min intersession intervals. Mice had to be capable of staying on the rod for 60 sec before proceeding to testing.

The three following consecutive days (Day 2, 3 and 4) corresponded to the **Testing phase**. Animals were placed on the rod then the apparatus was set to accelerate from 4 to 40 rpm in 5 min. The trial began when the device began accelerating and stopped when the subject fell off the rod. Latency to fall and the rpm associated were the parameters collected for analysis. The procedure was repeated for 4 trials separated by a 3 min intersession interval. After each session, the apparatus was deep-cleaned with ethanol 70% and water.

#### **b. Openfield**

The Openfield task was used to assess the ability of mice to deal with unescapable stressful situations. This test was carried out during adolescence (around PND-40), during adulthood (around 3-4 months) and later during aging (around 13-14 months). The setup consisted of a 60 cm (length [L]) x 60 cm (width [W]) x 40 cm (height [H]) nontransparent gray Plexiglas arena. The arena was indirectly illuminated with weak white light (30 lx in the center). Each mouse was put in a corner of the open field and allowed to explore the entire apparatus for 10 min. Video tracking software Ethovision (Noldus, Netherland) recorded and automatically analyzed the total distance travelled within the arena and the time spent in two distinct zones: the center and the periphery (sec). The apparatus was deep-cleaned between subjects using a 70% ethanol and water solution.

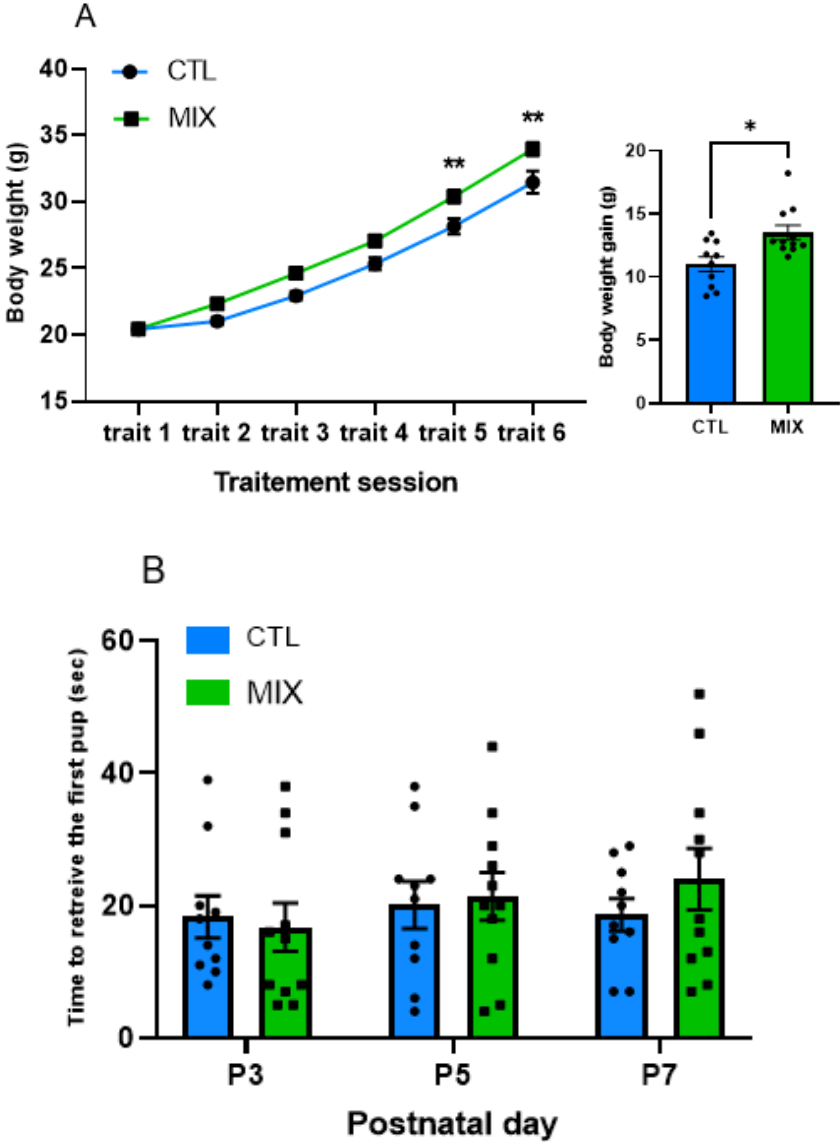
### **3.5 Statistical and analysis**

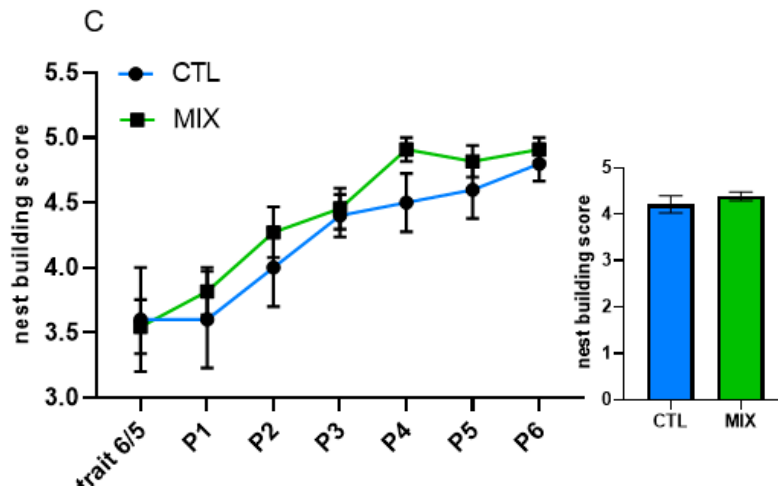
Data analysis was carried out using Graphpad Prism 9 (Graphpad Software, San Diego, CA, USA). Data are presented as mean±SEM for each experimental group. Each point corresponds to an individual. As we are assessing effects on small samples ( $n < 30$ ) and as our data do not follow a normal distribution, we analyzed them, using non-parametric procedures specially adapted for these kind of data. The two main advantages of this statistical procedure compared to a parametric approach are: These methods are not reliant on population assumptions, eliminating the risk of not satisfying prerequisite criteria. Moreover, they demonstrate improved power-efficiency compared to larger sample sizes. Independent two group comparisons were analyzed using the Mann–Whitney test. When more than two groups needed to be consider, the analysis was done using the Kruskal–Wallis “ANOVA-on-ranks” test, and then, when necessary, suitable post-hoc analysis (Dun test) was performed for intra group comparison. When an analysis required repeated measures, a two-way ANOVA with repeated

measures was performed. For the analysis of post-natal behavior, the Air Under the Curve (AUC) was measured for each individual and then a Man-Whitney test was performed for AUC comparison. P values  $\leq 0.05$  were considered statistically significant.

### III. Results

A maternal MIX exposure does not alter maternal behaviour but alters physiological parameters during pregnancy

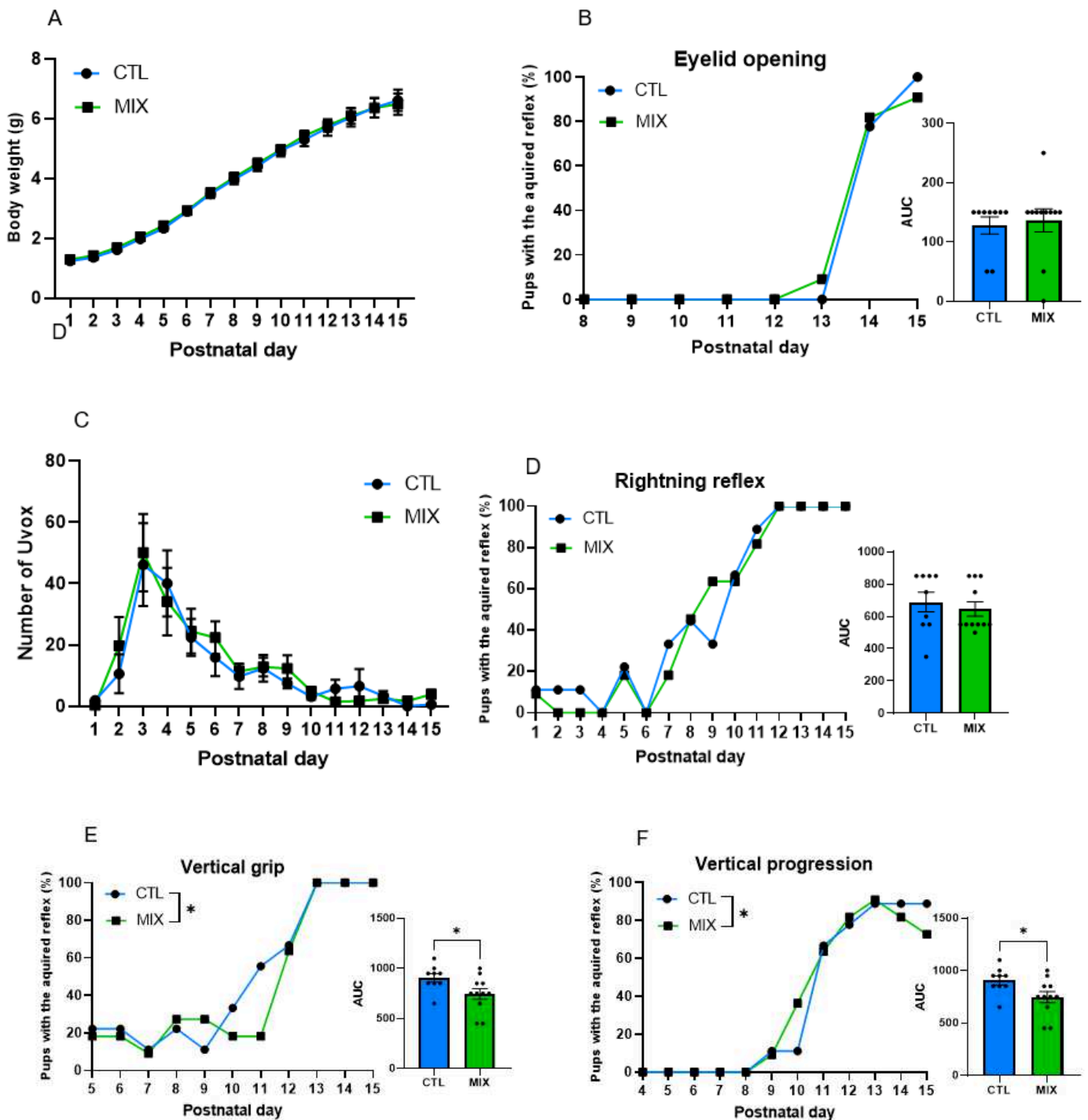




**Figure - 2** Effect of a chronic prenatal exposure to MIX (BMAA+GLY+GLA) on pregnant C57Bl/6 mice on body weight gain during pregnancy (A), on maternal behaviour in pup retrieving task (B) and nest building test (C). A prenatal chronic exposure to a mixture of environmental agents induced a significant increase of body weight intake during pregnancy (A). The latency to retrieve to the nest one isolated pup (B) or the ability to build a suitable nest were recorded (C). No significant differences were observed between groups (B, C). Data are expressed as mean  $\pm$ SEM. N=10 (CTL), N=11 (MIX). Comparison were made by a 2-Way ANOVA (A) and Man-Whitney (B, C) \* $p < 0,05$ , \*\* $p < 0,01$  compared to CTL group.

A prenatal chronic exposure to a “cocktail” of low doses of environmental toxicants induces a significant body weight intake during gestational period (Fig. 2A). However, maternal behaviour was not shown to be altered by a chronic MIX exposure, as time to retrieve the first pup at P3, P5 and P7 was not different between CTL and exposed dams (Fig. 2B). Additionally, the ability to build a suitable nest by the dams first days after delivery was not affected by maternal chronic exposure to the “cocktail” (Fig. 3C).

## Neurodevelopmental endpoints in offsprings are altered by a maternal chronic exposure of environmental agent's mixture

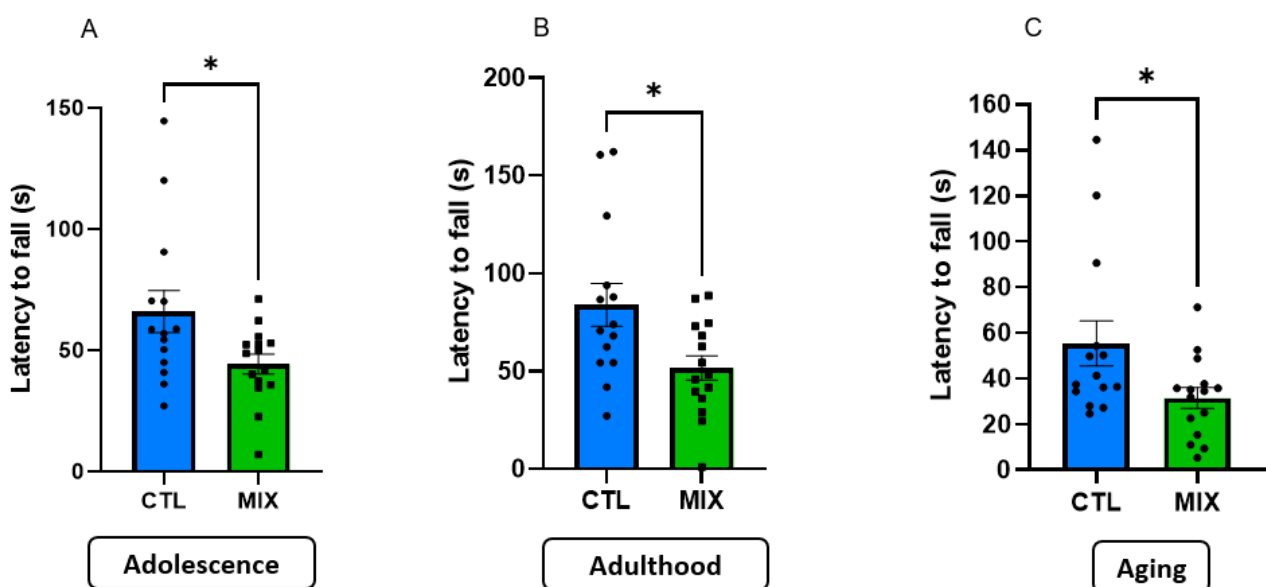


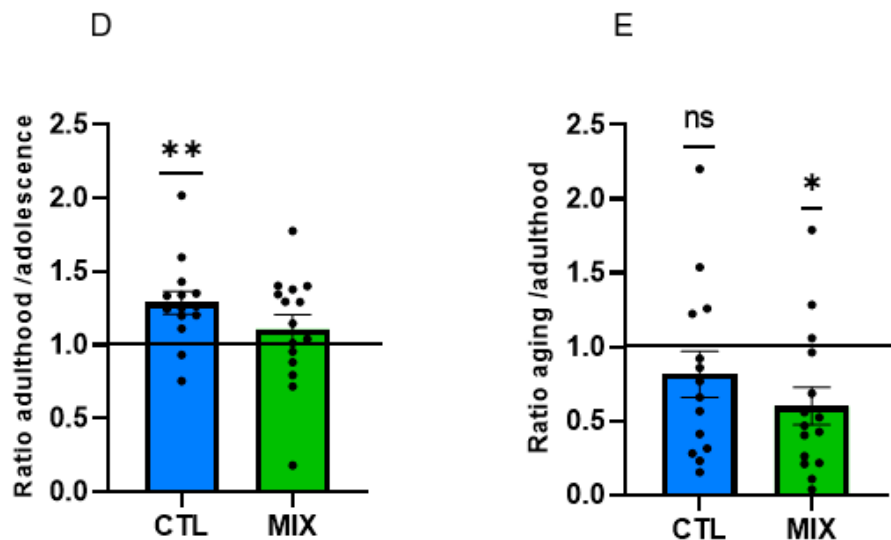
**Figure - 3** Neurodevelopmental endpoints in the offspring's following a chronic prenatal exposure to environmental toxicants mixture. Somatic growth parameters were assessed by measuring body weight gain (A) and eyelid opening day (B). Communicative skills were evaluated through ultrasonic calls measurement (C). Neuromotor development was investigated through the appearance of righting reflex (D), vertical grip (E) and vertical progression (F). Air Under the Curve (AUC) was measured for each point.

Data are expressed as mean  $\pm$ SEM. N=9 (CTL), N=11 (MIX). Comparison were made by a 2-Way ANOVA with repeated measure (A & C) and Man-Whitney for AUC comparison (B, D, E & F), \*p < 0,05 compared to CTL group.

A screening of developmental endpoints have been assessed by category informing on various aspects of offspring's neurodevelopment. Somatic growth and maturation were assessed through body weight gain from Post Natal day (PND) 1 to PND15 and eyelid opening from PND8 to PND15 as recommended. Data showed that prenatal MIX exposure did not alter early life body weight gain (Fig. 3A) or eyelid opening compared with CTL individuals (Fig. 3B). Communicative skills were addressed through the measurement of ultrasonic calls. The two-way repeated ANOVA revealed that MIX exposure did not alter the offspring's ability to emit ultrasonic calls in response to maternal separation (Fig. 3C). Neuromotor development was investigated through the appearance of various reflexes such as righting reflex, vertical grip, and vertical progression. Results showed that acquisition of righting reflex was not impacted by MIX exposure but AUC comparison revealed that maternal MIX exposure significantly slowed down offspring's ability to acquire vertical grip and vertical progression abilities (Fig. 3E & F).

### Chronic low-doses MIX maternal exposure triggers longitudinal and long-lasting motor coordination disturbances in offspring



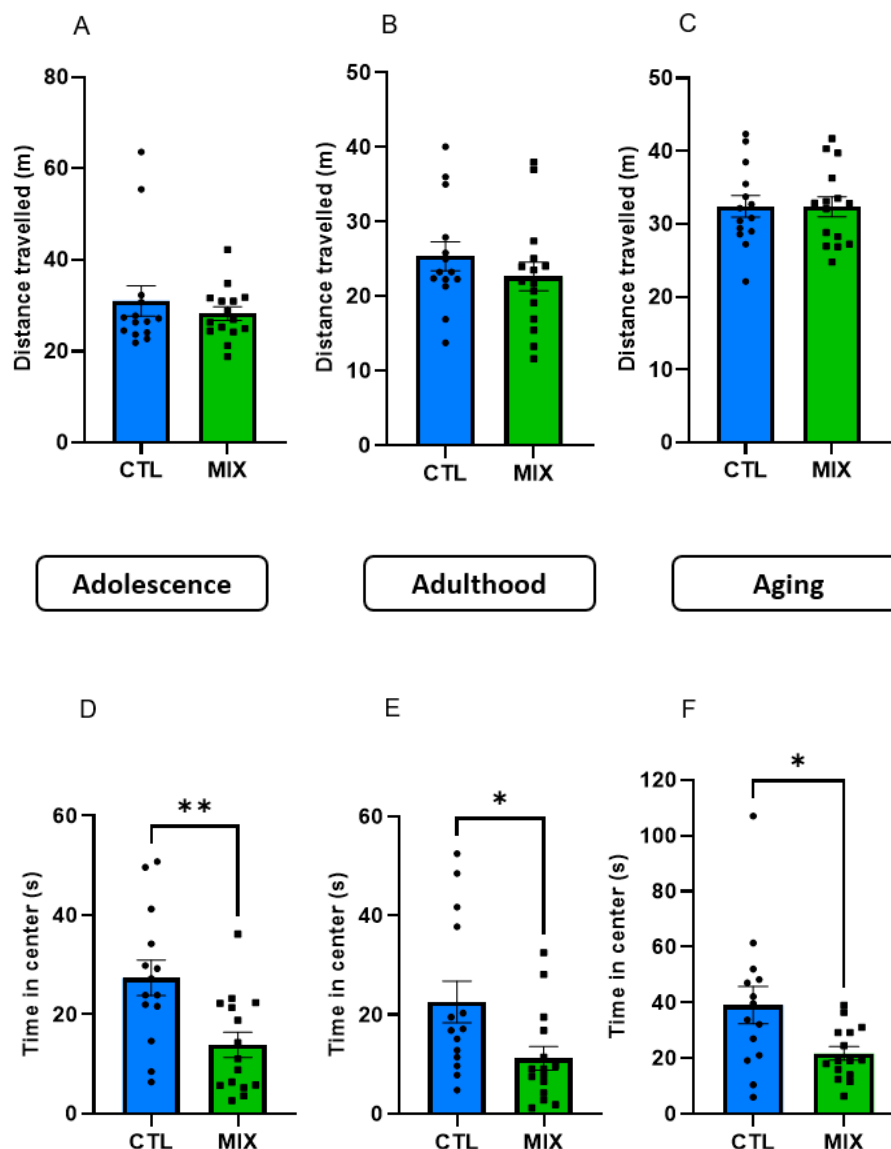


**Figure - 4** Effect of a chronic maternal environmental toxicants exposure on offspring's performances on the Rotarod test in adolescence (A), adulthood (B) and aging (C). A longitudinal evaluation of balance and motor coordination was performed in an accelerated mode of Rotarod test by measuring the latency to fall. Motor learning was represented in a ratio of latency to fall between adulthood and adolescence (D) and between aging and adulthood (E). Prenatal exposed MIX offspring's significantly fall earlier from the rod from adolescence to aging. Data are expressed as mean  $\pm$ SEM. N=14 (CTL), N=15 (MIX). Comparison were made by Man-Whitney  $*p < 0,05$ , compared to CTL group.  $*p < 0,05$ ,  $**p < 0,01$  (One sample Wilcoxon) compared to an hypothetical value of 1.

The balance and motor coordination were assessed in an accelerated mode in the rotarod task by measuring the latency to fall. During the adolescence period, offspring prenatally exposed to a "cocktail" containing low dose of BMAA, GLA and GLY fell significantly faster from the rod compared to CTL group (Fig. 4A). Later, during adulthood and aging, the same profile was observed about this parameter, as MIX individuals exposed through their mother displayed a significant decrease of the latency to fall from the rod, suggesting a long-term effect (Fig. 4B & 4C). In order to evaluate motor learning abilities between adolescence and adulthood first, and between adulthood and aging, the ratio of latency to fall during adulthood and adolescence, and during aging and adulthood have been calculated. Only CTL group expressed a motor learning as the ratio was significantly higher to the hypothetical value of 1. Latency to fall during adolescence and adulthood are not statistically difference as the ratio is not different to 1 in mice exposed to MIX prenatally (Fig. 4D). However, mice from MIX group displayed a ratio significantly lower than 1, between latency to fall in adulthood and

aging, illustrating here a drop of motor coordination performances (Fig.4E). Thus, a prenatal chronic exposure to low doses of a “cocktail” of environmental toxicants induce a impairment of motor coordination performance, and motor learning with disruption occurring in adolescence persisting into adulthood and aging, illustrating here a drop of motor coordination performances.

### Chronic low-doses MIX maternal exposure triggers longitudinal and long-lasting emotional behaviour disturbances in offspring



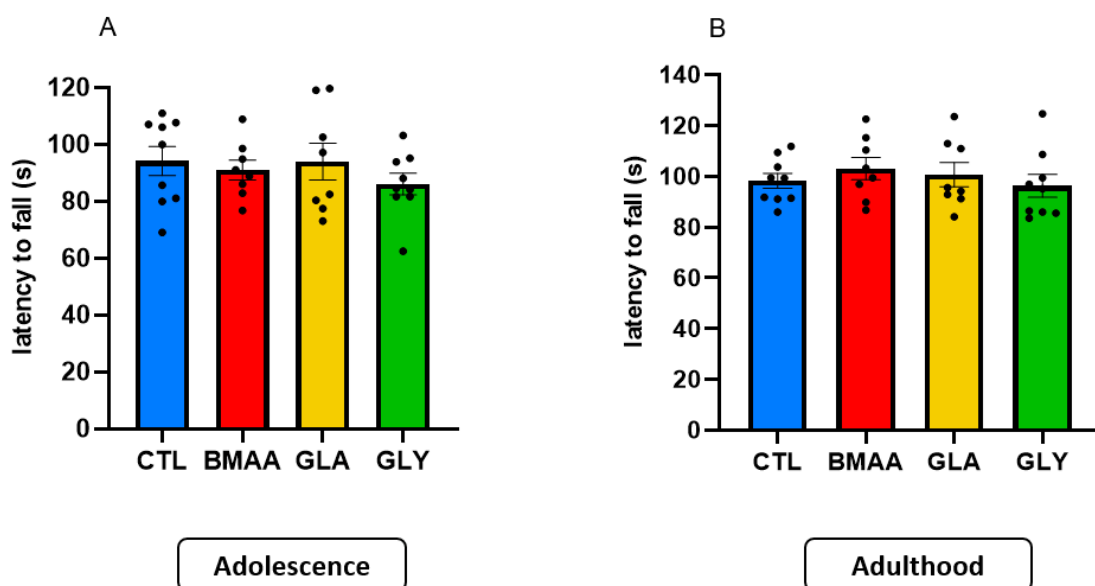
**Figure - 5** Evaluation of emotional parameters (time spend in the center) and locomotor activity during adolescence (A & D), adulthood (B & E) and during aging (C & F) in the Openfield. Maternal environmental toxicants exposure did not influence offspring's locomotor activity neither in



adolescence, adulthood or aging (A, B & C) but triggered longitudinal and persistent emotional behaviour impairments in offspring's (D, E & F) in the Openfield task. . Data are expressed as mean  $\pm$ SEM. N=14 (CTL), N=15 (MIX). \*p < 0,05, \*\*p < 0,01 (Man-Whitney) compared with Sal/CTL group

Evaluation of locomotor activity showed that individuals exposed to MIX prenatally did not present any statistical differences with control group on the total distance travelled, from adolescence to aging through adulthood (Fig. 5 A, B, C). In order to evaluate anxiety state, time spent in central zone has been recorded. During adolescence, male mice from MIX exposed group spent significantly less time in the central zone of the openfield (Fig. 5D). Later during adulthood and aging, MIX exposed individuals still spent less time in the central zone compared to CTL group (Fig. 5 E, F). Thus, a prenatal chronic exposure to low doses of a “cocktail” of environmental toxicants induce an impairment of emotional behaviour without modify general locomotor activity.

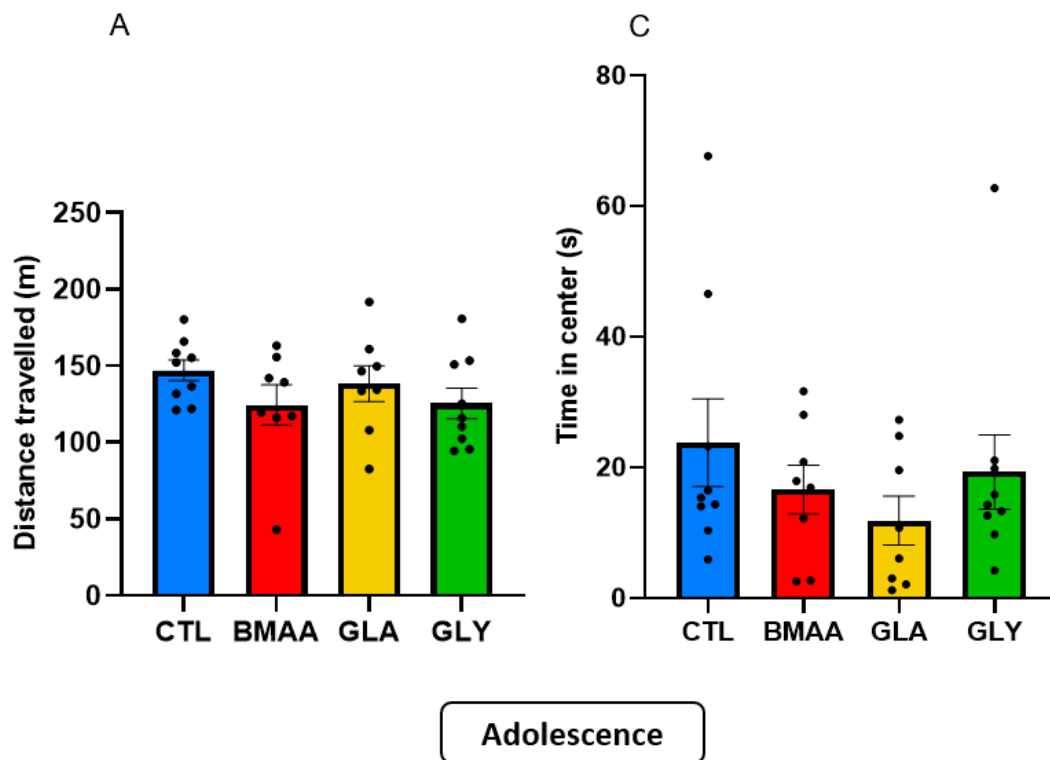
### Motor coordination in offspring's is not affected by a prenatal chronic environmental agents exposure

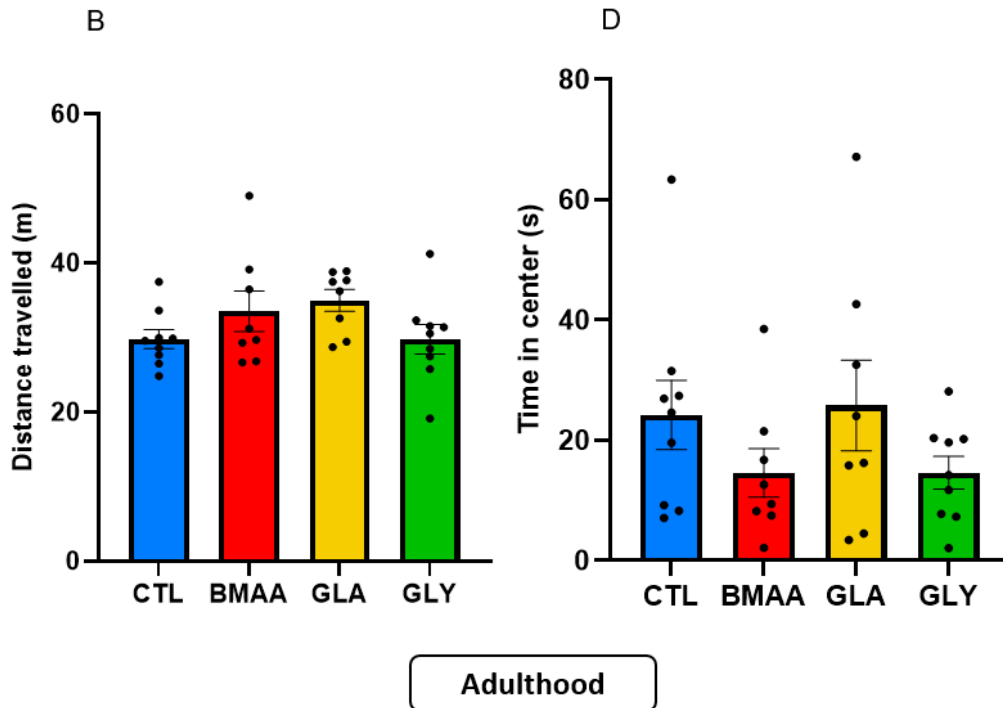


**Figure - 6** Effect of a chronic prenatal exposure to exposure to BMAA (50 mg/kg), GLA (0,2 mg/kg) or GLY (5 mg/kg) on the Rotarod test in adolescence (A) and adulthood (B). The balance and motor coordination of young and adult male mice were assessed in an accelerated mode of Rotarod test by measuring the latency to fall. No significant differences were observed between groups. Data are expressed as mean  $\pm$ SEM. N=9 (CTL), N=8 (BMAA), N=8 (GLA), N=9 (GLY). Comparison was made by Kruskal Wallis compared to CTL group.

The balance and motor coordination were assessed in an accelerated mode of Rotarod test by measuring the latency to fall. During the adolescence period, offspring prenatally exposed to either BMAA, GLA or GLY did not display an impairment of motor coordination as the latency to fall was not different compared to CTL group (Fig. 6A). The same profile is observed later in adulthood where no differences were observed compared to CTL group (Fig. 6B). Data showed that a chronic prenatal exposure to low doses of environmental toxicants as a single compound did not induce an impairment of motor coordination performance, neither in adolescence nor in adulthood.

### Emotional and locomotor behaviour in offspring's is not affected by a prenatal chronic environmental agents exposure





**Figure - 7** Evaluation of emotional parameters (time spend in the centre) and locomotor activity during adolescence (A & C) and adulthood (B & D) in the Openfield. Maternal environmental toxicants exposure did not influence offspring's performances on locomotor activity (A & B) and emotional behaviour (C & D) in the Openfield task. Data are expressed as mean  $\pm$ SEM. N=9 (CTL), N=8 (BMAA), N=8 (GLA), N=9 (GLY). Comparison was made by Kruskal Wallis compared to CTL group.

Anxiety state and locomotor activity were tested by the open-field task. During adolescence, mice prenatally exposed to either BMAA, GLA or GLY spent similar time duration in the centre of the arena and travelled the same distance compared to CTL group (Fig. 7A, C). During adulthood, offsprings exposed to either BMAA, GLA, GLY through their mother did not display modification of general locomotor activity. The total duration spent in the central zone of the open-field is not impacted in these groups compared to control individuals (Fig. 7B, D). Thus, a maternal chronic exposure to low doses of environmental toxicants does not impaired emotional behaviour and locomotor activity in these conditions.

## IV. Discussion

Exposure to environmental xenobiotics, such as pesticides or toxins, has already been associated with neurodevelopmental toxicity, leading to neurological disorders later in life (London et al., 2012; Richardson et al., 2019). Typically, individuals are exposed to combinations of different compounds. However, the majority of studies focus their analysis on the effects of single compound at a time. Exposure during development to mixtures of pesticides can lead to additive effects or interactions, not observed with a single compound. The present work aimed to explore the concept of “cocktail effect”. For that, we exposed chronically pregnant mice, during the gestational period, to low doses of either BMAA, GLY or GLA alone or as a cocktail by intranasal route. Our results suggest that a prenatal exposure to low doses of our xenobiotics alone is not sufficient to disturb behavioral motor and emotional performances in adolescence and later in adulthood. However, exposure to a mixture containing low doses of a pesticide, an herbicide and a cyanotoxin leads to significant behavioral changes in offspring from the post-natal period to adolescent, adult and aging stages. Our work demonstrates that a maternal exposure to a pollutant mixture induces long lasting neurological disorders. This suggests a synergistic and additional effect in line with the “cocktail effect”.

Over years, it has been well documented that maternal environment plays a crucial role in the development of neurological disorders such as neurodevelopmental pathologies (Han et al., 2021). In fact, the maternal environment directly influences fetal development during pregnancy. Exposure to toxic substances, such as pesticides or neurotoxins, can have detrimental effects on the developing fetus's nervous system (Suwannakul et al., 2021). Moreover, it is known that disturbances in maternal behavior can lead to behavioral deviances in the offspring (Moura et al., 2023). Since our exposure was made directly through the mother, it was important to first evaluate the effects of such molecules on the physiological parameters of females as well as their ability to care for their offspring. Under our experimental conditions, we demonstrated that maternal behavior was not affected by exposure to a cocktail of xenobiotics. However, a significantly greater weight gain was observed in these females during the exposure period. Interestingly, certain pesticides are considered endocrine disruptors, meaning they can interfere with the normal functioning of the hormonal system (Ahn & Jeung, 2023; Leemans et al., 2019). Some pesticides can disrupt the hormonal balance involved in body weight regulation, potentially leading to excessive weight gain (Kitamura et al., 2006).

Furthermore, some epidemiological studies suggest that pesticide exposure can alter the energy metabolism in pregnant women (Bonvallot et al., 2013). Pesticides can affect fat metabolism, resulting in excessive accumulation of body fat and weight gain. However, further research is essential to better understand the underlying mechanisms involved and establish more precise links between exposure to this type of xenobiotic and weight gain during pregnancy.

Regarding our xenobiotics selection, it has previously been established that exposure to BMAA, GLA, or GLY can have a significant impact on neurodevelopment. (E. T. Chang et al., 2023a; Newell et al., 2022b). In our experimental conditions, using low doses of these compounds, we observed that maternal exposure to low doses of BMAA, GLY, or GLA did not induce deleterious effects on motor or emotional behavior in the offspring, during adolescence or adulthood. Usually, studies demonstrating such effects use high doses. For example, it has been shown that postnatal exposure to BMAA induces motor and cognitive disruptions in adulthood, but at a dose of 600 mg/kg. (Karlsson et al., 2009a). Similarly, de Oliveira and colleagues have shown that maternal exposure to GBH induces behavioral disruptions in the offspring, associated with neurodevelopmental disorders, at a dose of 50 mg/kg (de Oliveira et al., 2022a). Indeed, few studies have explored the effects induced by low-dose exposure to these agents. For example, studies using a murine model, have demonstrated that exposure to low doses of BMAA either does not induce any effects or only modest effects on behavioral performance, both in adults and offspring (Cruz-Aguado et al., 2006; Laugeray et al., 2018). Consistent with these observations, previous studies conducted by our team have shown that exposure to GLA at 0.2 mg/kg is not associated with behavioral disturbances in the offspring (Herzine et al., 2016; Laugeray et al., 2014). Taken together, these results demonstrate that these xenobiotics do not induce major effects at the doses studied.

However, the evaluation of the effects of these compounds as a cocktail shows, this time, alterations in neurodevelopmental trajectories in offspring exposed during the gestational period. Starting from the postnatal period, early disruptions in the establishment of certain sensorimotor skills, such as vertical grip, have been observed in offspring exposed to the mixture through their mothers. During adolescence, these same individuals exhibit disturbances in motor coordination, motor learning, as well as an increase in anxious behaviors. These behavioral disruptions are still observed in adulthood and later during aging. These

results demonstrate, particularly at low doses, that concomitant exposure to multiple environmental agents is often necessary to induce early and long-lasting behavioral changes. This is consistent with previous *in vivo* studies focusing on Paraquat (pesticide) and Maneb (fungicide), which showed that exposure, both during the perinatal period and in adulthood, induced synergistic or additive effects, particularly on locomotor activity and dopamine levels in the brain, with the mixture but not with the compounds taken individually (Cory et al., 2005; Thiruchelvam et al., 2000b). Another study evaluated the effects of a mixture containing 11 pyrethroids and revealed that when compounds, at sublethal doses, were administered as a mixture, a significant decrease in locomotor activity in mice was observed. (Wolansky et al., 2009a). Taking together, these results support the importance of considering the cocktail effect, suggesting the involvement and disruption of different pathways, which call for further investigation in future research.

In conclusion, our findings demonstrate that maternal exposure to a low dose of xenobiotics as a cocktail, induces a synergistic effect of these compounds on motor performance, ranging from motor coordination to motor learning. To our knowledge, our study is the first to longitudinally evaluate the effects of such a mixture from the gestational period to aging on the emergence of these types of symptoms. These discoveries raise an important public health issue in terms of the evaluation of the toxicological effects of various molecules. Further clinical, pre-clinical, and epidemiological investigations are necessary to understand the impact of multiple exposures to various insults. It is crucial to consider the cumulative and or synergistic effects of these exposures on developmental neurotoxicity. This emphasizes the need for comprehensive regulatory assessments that take into account “cocktail effect”. Additionally, these findings provide a strong basis for future toxicological research aimed at defining doses without detrimental effects, taking into account the interaction with various compounds. Ultimately, this knowledge can contribute to the development of new adverse outcome pathways.

# Part 3: GENERAL DISCUSSION

## The involvement of pesticides in the environment and human health and the controversy surrounding due to economic factors

For several decades, the use of chemical products in conventional agriculture has raised concerns and worries. Indeed, the use of these compounds, such as neonicotinoids and organophosphates, has significant repercussions due to their direct or indirect impact on the environment or human health (especially due to the varying selectivity of used products).

In the context of my thesis, two herbicide pollutants resulting from human activity have been studied: glyphosate and glufosinate ammonium. Due to suspicions of their effects on human health, the use of glufosinate has been banned in France since 2017 (Anses, 2017). In January 2019, a proposal law on agriculture and food regarding the use of glyphosate was adopted. It provided for the prohibition of glyphosate use in France by 2023. However, even if France prohibits the use of these compounds, it is important to consider that French regulations differ from European regulations. Thus, a pesticide that is prohibited in France may still be present in the food we consume, originating from neighboring countries that continue to use them.

Furthermore, some compounds that have been banned for over 20 years still remain controversial due to concerns about their effects on the environment and health. They can have harmful effects on terrestrial and aquatic ecosystems, contaminate soils, surface waters, and groundwater, leading to a decrease in biodiversity, the death of non-target organisms, and disruption of food chains. Some pesticides persist in the environment for long periods, increasing the risks of accumulation in living organisms. Moreover, they can present risks to human health, particularly for farmers, agricultural workers, and people living near treated areas. Exposure to pesticides has been associated with health issues such as skin irritations, respiratory problems, neurological disorders, hormonal disruptions, and cancers. There are also concerns about chronic exposure to pesticide residues in food and potential long-term health effects. This raises concerns about the impact of these residues on human health, especially when found in commonly consumed fruits, vegetables, and other food items. Strict regulations are in place to limit pesticide residues in food, but risk perception varies.

This raises questions since despite the proven harmful effects of these compounds, some active substances struggle to be banned. It is important to note that the controversy surrounding

pesticide use is complex, and opinions can vary depending on environmental, economic, and social perspectives. According to the United Nations of Human Rights Council, the pesticide market represents over 47 million euros per year. Large multinational corporations, such as Bayer/Monsanto, benefiting from these profits, argue that the withdrawal of pesticides in agriculture would lead to global famine due to a significant decrease in yields. However, despite the economic advantages offered by the use of such compounds, it should be necessary to consider the environmental and health costs associated with their use. Negative impacts on the environment, such as water pollution and loss of biodiversity, can result in significant costs in terms of ecosystem restoration and nature conservation. Furthermore, costs related to human health problems associated with pesticide exposure can include healthcare, productivity losses, and social impacts.

### **Evaluation of the toxicity of amino acid analogues: limitations of existing models and uniqueness of our study**

On a human scale, exposure to different compounds can occur at various levels. In the case of pesticides, human beings can be exposed acutely, including unintentional poisoning or suicide (intentional), but also chronically. This can be the case for agricultural populations in rural areas, neighboring populations exposed through the air, as well as consumers of treated products exposed orally. Additionally, fetuses and young children are particularly vulnerable to the effects of pesticides due to their developing immune system and heightened sensitivity during these stages of life. In toxicology and pharmacology studies, it is important to consider the routes of exposure and the relevant time periods. The brain is one of the targets of exposure to various xenobiotics. Some substances can have harmful effects due to their ability to cross body's protective and natural barriers, such as the blood-brain barrier or the placental barrier, and can therefore be present at the cerebral level or in the amniotic fluid (Mulder et al., 2019).

However, toxicological assessments rarely take into consideration the multifactorial aspect with which we are confronted on a daily basis. Generally, regulations establish threshold limits below which substances are judged to be harmless. However, according to Professor Barouki, "the scientific foundations of these reference thresholds are contested, as certain substances can have notable effects even at very low doses." A concrete example is that of endocrine disruptors, which can have harmful effects during vulnerable periods, such as the early years of a child's life (Ghassabian et al., 2022; Palanza et al., 2021). It is possible that some reference



values established from non-exhaustive regulatory tests do not provide sufficient threshold concentrations for adequate protection. These tests are considered non-exhaustive because they are limited to a narrow range of doses and do not take into account all toxicity mechanisms, such as epigenetic modifications, for example. Another example refer to vulnerability windows, particularly during the physical development of a child. This process relies on sensitive mechanisms that can be altered by exposure to pollutants. These disruptions could lead to impaired organ function, which could promote the onset of long-term diseases. Therefore, toxicology must "develop analyses that assess risks in vulnerable individuals and during the most sensitive developmental stages." Another issue lies in the considerable time lapse between exposure to a toxic substance and the appearance of health effects, which can span several decades. In some cases, these effects may even manifest in subsequent generations that were not directly exposed. For instance, long-term effects can be attributed to persistent pollutants that are difficult for the body to detoxify and accumulate in adipose tissues, thus constituting continuous internal sources of pollutant exposure, as well as hidden effects that may have consequences later on.

Another important point to address is the concept of multiple exposures. Usually, regulations consider substances individually, while the population is exposed to countless mixtures whose compositions change over time. When substances have similar modes of action, current knowledge suggests that the addition of doses can reasonably predict the effects of a mixture. However, when modes of action differ, the toxicities of substances in a mixture can sometimes combine synergistically or neutralize each other (de Souza et al., 2019; Gamet-Payrastre, 2019).

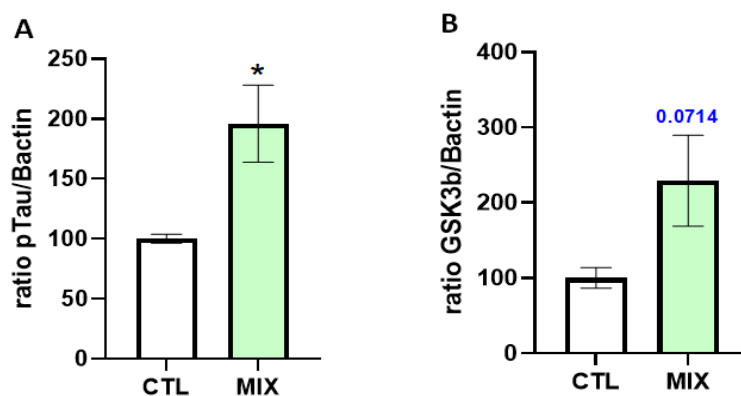
All these limitations highlight a number of doubts, emphasizing the importance of considering all these observations, such as bioamplification, potential cocktail effects, taking into account possible additive or synergistic interactions, as well as the exposure period. This is the unique aspect of the model that we attempted to establish in this study. Indeed, the main objectives were to evaluate the *in vivo* effects induced by three amino acid analogues: a cyanotoxin (BMAA), an herbicide (glufosinate ammonium), and a pesticide (glyphosate), taking into account their concomitant presence in the environment. During the development of the ANR project, in which this thesis is part of, there were no studies evaluating the potential interactions of BMAA with two herbicides. Furthermore, the notion of inflammatory sensitization played an important role in this project, highlighting an important concept known

as "multiple hits." Therefore, we attempted to establish a multifactorial model that takes into account the previously mentioned dimensions: **multiple exposures** (in several "hits" and/or concomitantly as a mixture), **early exposure during the perinatal period** (highlighting the importance of sensitive developmental windows), and **chronic exposure at low doses**. Another major characteristic was the behavioral evaluation with a longitudinal monitoring. Indeed, the effects of such exposure were evaluated during early (postnatal) periods, adolescence, adulthood, and later during aging, when it was possible.

As part of our study, I was able to demonstrate that an asymptomatic inflammatory event (pre- or postnatal) sensitized exposure to these xenobiotics, whether in combination or separately, thereby inducing early and lasting behavioral effects in the offspring. We also showed that exposure to a mixture containing low doses of BMAA, GLA, and GLY during the perinatal period induced synergistic, detrimental, and long-lasting effects in the offspring. Indeed, exposure to low doses of these different compounds individually did not show such disruptions in the offspring, suggesting this synergistic effect. However, in certain *in vivo* cellular criteria, after gestational exposure, the synergistic effect was not observed. Evaluation of different behavioral domains revealed, in particular, disturbances in motor performance (locomotion and motor coordination), as well as disruptions in emotional behavior. It had already been shown that exposure to such compounds leads to this type of behavioral disruption. Studies assessing postnatal exposure to BMAA (600 mg/kg) demonstrate that it leads to motor and cognitive disturbances in adulthood (Karlsson et al., 2009b). Similarly, various studies conducted by Ait Bali and his team have shown that exposure to Glyphosate Based Herbicides (GBH), either in young adults or during the perinatal period, induces behavioral disruptions, particularly locomotor activity disturbances and delays in the acquisition of certain motor reflexes (Ait Bali et al., 2017b; Ait-Bali et al., 2020b). Recently, regarding glufosinate ammonium, a study showed that maternal exposure to GLA (2 mg/kg) also induces locomotor activity disturbances in the offspring (Dong et al., 2020b). These types of symptoms were suspected to be caused by exposure to this type of xenobiotic because several epidemiological studies have highlighted motor changes following exposure to BMAA (Newell et al., 2022a; Riancho et al., 2018) or glyphosate (or GBH) (E. T. Chang et al., 2023b). More largely, an association with pathologies related to motor disturbances has been suspected. For example, BMAA has been found in the spinal cord of individuals with ALS (Pablo et al., 2009b), supporting

studies conducted in non-human primates (Davis et al., 2020, 2021). Indeed, over the past few decades, BMAA has been associated as an environmental risk factor in the etiology of neurodegenerative diseases such as ALS or Parkinson's-like disease, with one of the major symptoms being motor impairment (Caller et al., 2018; Cox et al., 2018; Pablo et al., 2009b). Similarly, an increasing amount of epidemiological data consider glyphosate exposure as a potential risk factor for the development of Parkinson's-like diseases (Eriguchi et al., 2019; Samsel & Seneff, 2015b).

However, there are limitations to be considered regarding the observed effects. Indeed, on their own, they do not allow us to assert that they are related to a specific pathology associated with motor disorders. Our behavioral analysis is insufficient to characterize the specific type of disorder involved. Therefore, for better characterization, it would be necessary to determine the origin of the impairment (cerebral, spinal, or neuromuscular) as well as investigate characteristic biomarkers of such alterations. In the literature, the level of phosphorylation of Tau protein is recognized as a marker for neurodegenerative diseases such as ALS, Alzheimer's, or Parkinson's (Karikari et al., 2020; Rong et al., 2021; Strong et al., 2020). Furthermore, the pathways involved in such disorders would involve GSK3 $\beta$ , whose activity phosphorylates Tau (Choi et al., 2020; Samim Khan et al., 2023). Changes in these factors have been observed in ALS SOD-1 models, including increased phosphorylation levels of Tau protein and upregulation of GSK3 $\beta$  activity (Koh et al., 2007). Interestingly, *in vivo* studies have shown that exposure to BMAA induces similar modifications (de Munck et al., 2013). This seems to be in line with the observations we made using a mixture containing a BMAA dose up to 7 times lower than their dose. Preliminary data quantifying the protein level of phosphorylated Tau protein and GSK3 $\beta$  have been obtained from the brains of offspring exposed to the MIX during the prenatal period. As indicated by the preliminary results in Figure 23, the protein levels of phospho-Tau and GSK3 $\beta$  are higher in the brains of MIX individuals at P5 (Figure 23). Another crucial factor in the emergence of such pathologies is the role of TDP-43. Indeed, an accumulation of TDP-43 has been identified as a biomarker for neurodegenerative diseases such as ALS (Yu et al., 2020). The TDP-43 model, along with the SOD1 model, is characteristic in the study of ALS (Joyce et al., 2011).



**Figure 23** - Evaluation of protein levels of phosphorylated Tau protein and GSK3 $\beta$  by Western blot in the brains of offspring at P5 following prenatal exposure to MIX. N = 4 CTL et N=4 MIX, non parametric comparison (Mann-Whitney). \* p<0.05

The identification of these biomarkers in individuals presenting disorders that could be associated, particularly with motor neuron diseases (MND), would allow for better characterization and, therefore, strengthen the link between exposure, behavioral disorders, and resemblance to characteristic neurodegenerative pathologies.

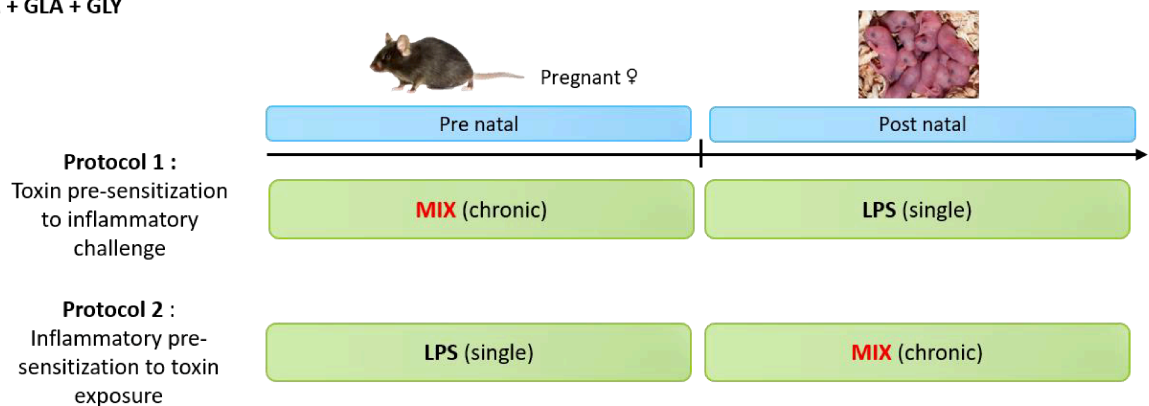
The disorders observed in the context of these exposures are not only present in individuals exposed to the cocktail but also in those subjected to the double hit (asymptomatic inflammatory challenge and exposure to xenobiotics alone or in mixture). This raises questions about the data observed in the literature, which often show effects only with individual molecules at high doses. Furthermore, our data have shown the importance of considering not only the concept of a cocktail but also multiple hits. Increasingly, studies tend to incorporate these concepts into their experimental strategies. This cocktail effect has been particularly investigated in the context of pesticide exposure. *In vivo* studies examining another pesticide (paraquat) and a fungicide (maneb) have shown that exposure during both the perinatal period and adulthood induces effects, particularly on locomotor activity and dopamine levels in the brain, with the mixture but not with the individual molecules. In a postnatal model, a cumulative neurotoxic effect is observed, with the effects of the mixture being greater than those of the separate molecules (Cory et al., 2005; Thiruchelvam et al., 2000b). Another study evaluated the effects of a mixture containing 11 pyrethroids and revealed that when compounds at sublethal doses were administered as a mixture, there was a significant decrease in locomotor activity in mice (Wolansky et al., 2009a). Another study evaluated the effects of a

mixture containing 11 pyrethroids and revealed that when compounds at sublethal doses were administered as a mixture, there was a significant decrease in locomotor activity in mice (Wolansky et al., 2009b). These studies emphasize the importance of considering the cocktail effect and highlight the limitations of toxicological studies in defining doses without effects, without taking into account interactions with other compounds. They also underscore that a single risk factor is not always sufficient to cause lasting disturbances, directly referring to the multiple hit hypothesis. The behavioral effects related to this double hit are consistent with recent literature data showing that maternal inflammation sensitizes postnatal exposure to THC, leading to behavioral deficits (Guma et al., 2022). A comprehensive assessment of the toxicological effects of pesticides and biotoxins is necessary, and further clinical, preclinical, and epidemiological studies taking into account multiple exposures (cocktail and/or multiple hits) to various stressors need to be conducted.

### The prenatal period, a particularly sensitive window for xenobiotic exposure

As part of my work, we were able to test different experimental procedures by varying the exposure periods (Figure 24).

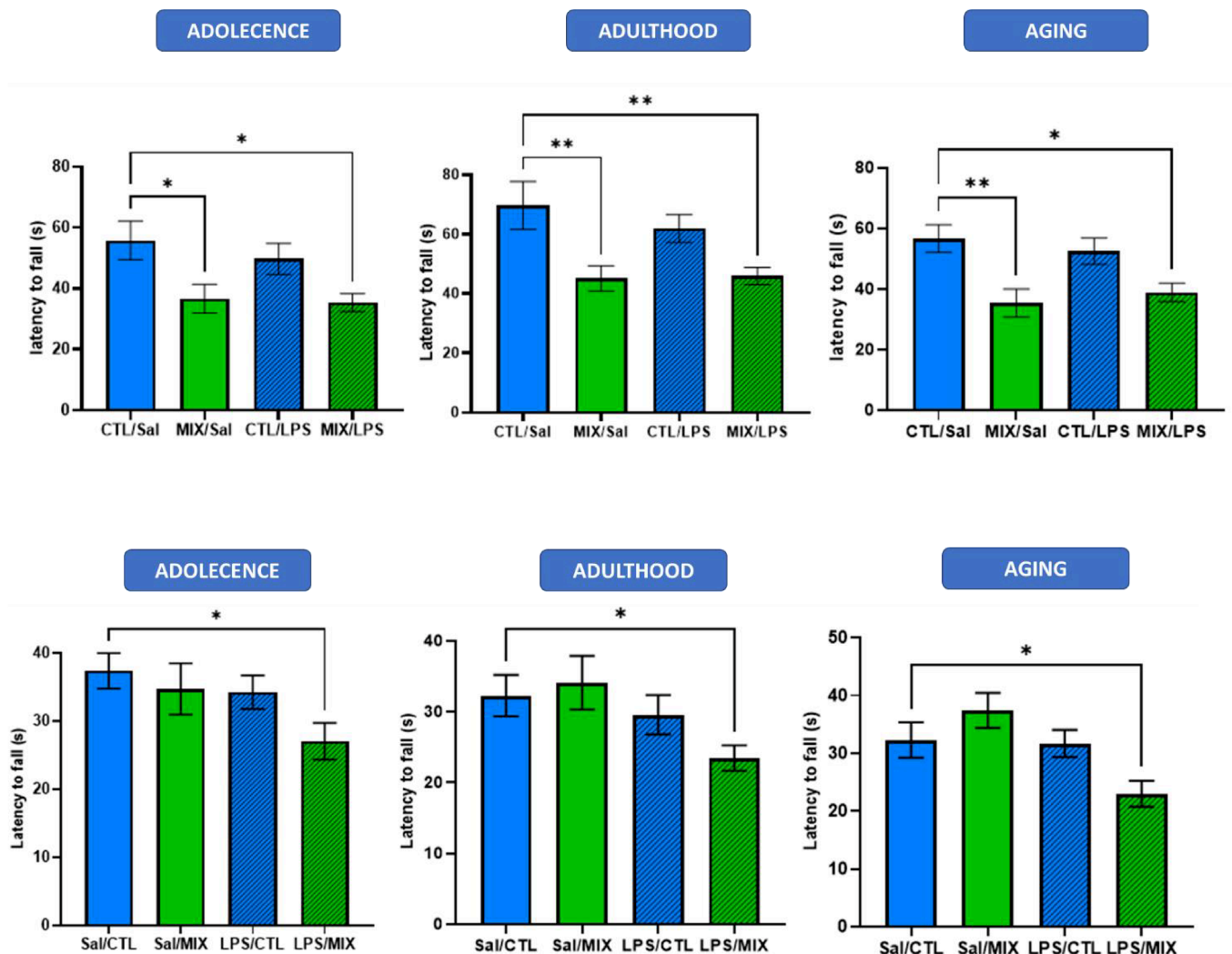
**MIX = BMAA + GLA + GLY**



**Figure 24** – Schematic version of experimental procedure used in this study. We investigated the effects of either a toxin pre-sensitization to inflammatory challenge or an Inflammatory pre-sensitization to toxin exposure.

We specifically aimed to determine whether prenatal exposure to the cocktail would sensitize an inflammatory challenge occurring during the postnatal period, or conversely, if maternal inflammatory challenge would sensitize to subsequent exposure to xenobiotics. My data indeed showed that prenatal exposure to LPS sensitized postnatal exposure to toxins (Oummadi A et al.,

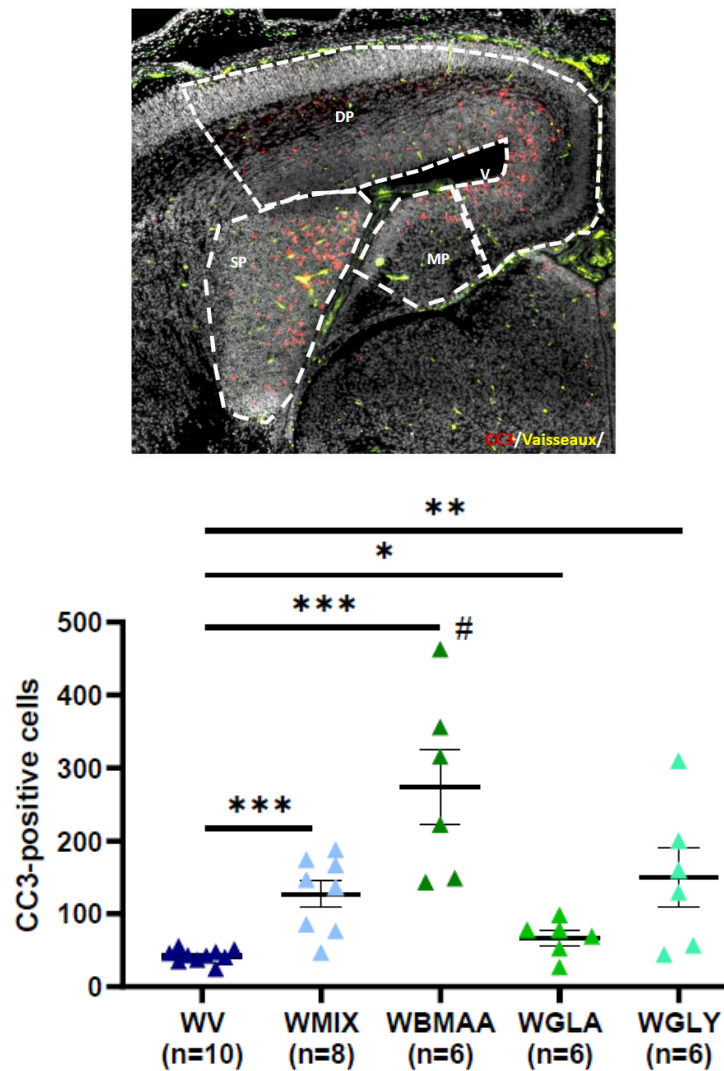
2023). In fact, postnatal exposure or sensitization alone was not sufficient to induce effects in the offspring. Conversely, when the pregnant female was exposed to the MIX, the offspring exhibited behavioral disturbances that were not exacerbated by an inflammatory episode during the postnatal period. We found that when the MIX was administered during the prenatal period, it led to motor disturbances in the offspring. However, when administered during the postnatal period, no motor effects were observed in the offspring. The same applies when an inflammatory episode is added during the exposure protocol. When this episode occurs during the postnatal period, there are no synergistic effects with prenatal exposure to the compounds. However, during the prenatal period, it sensitizes to subsequent exposure to the compounds (Figure 25). In this case, we do observe a synergistic effect.



**Figure 25** – Evaluation of a toxin pre-sensitization to inflammatory challenge (up) and an inflammatory pre-sensitization to toxin exposure (down) on motor coordination in Rotarod. N=14 (CTL/Sal), N=15

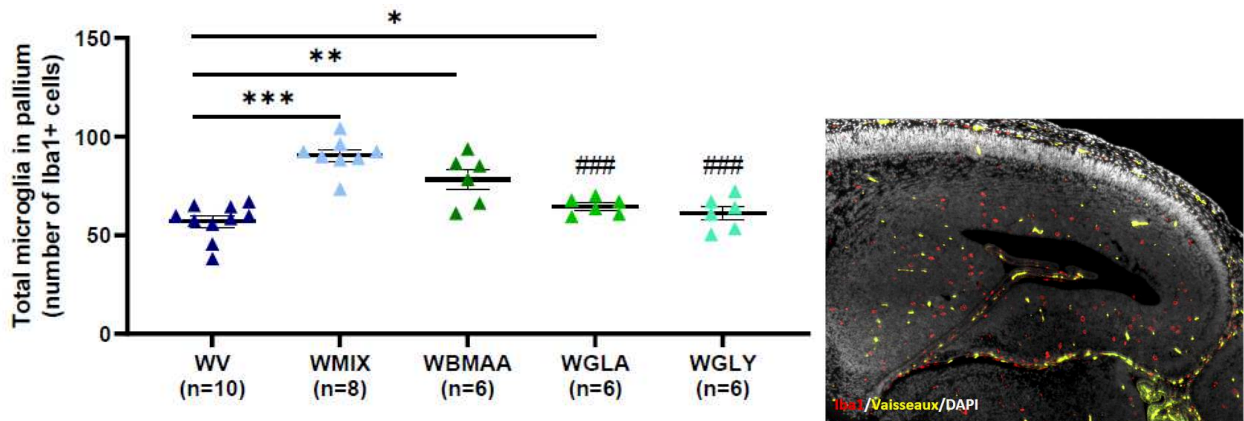
(MIX/Sal), N=14 (CTL/LPS), N=14 (MIX/LPS) (up) and N=12 (Sal/CTL), N=12 (Sal/MIX), N=12 (LPS/CTL), N=13 (LPS/MIX) (down). Kruskal Wallis comparison to CTL group. \* p<0.05, \*\* p<0.01

Based on these results, it appears not only that the cocktail effect is significant, but also that the exposure period is, and particularly the gestational period as it is especially sensitive to the exposure of such combinations of exposures.



**Figure 26** – Total number of CC3-positive cells in the whole brain of embryos exposed during the prenatal period.

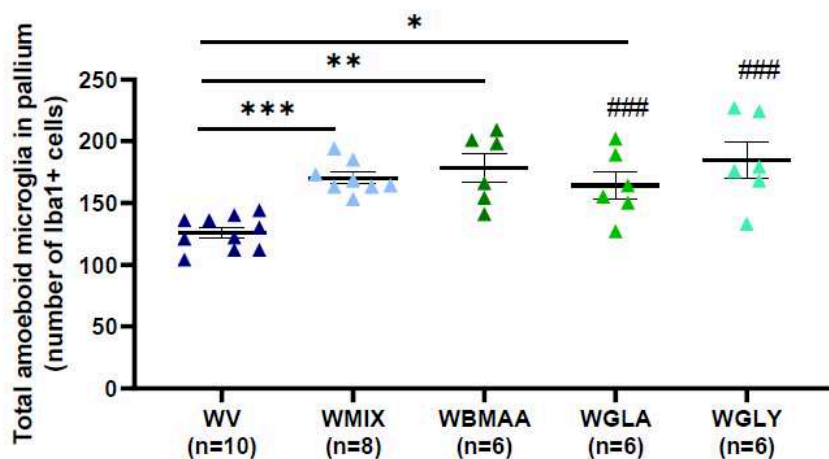
Other *in vivo* data obtained by ANR partners confirm this hypothesis. Indeed, although François Boussin and his team do not highlight synergistic effects of the compounds among themselves at the doses studied, they nevertheless observe harmful effects on cell death phenomena as well as on the cell cycle of neurogenic niches (Figure 26).



**Figure 27** – Number of IBA1-positive cells in the whole brain of embryos exposed during the prenatal period.

They confirm that the exposure window (ED 15.5) is crucial for observing harmful effects. They also observed a proliferation of microglial cells in embryos following exposure to MIX and BMAA (Figure 27).

Their immunohistochemistry analyses, conducted on whole-brain sections of mouse pups exposed prenatally, also identified that MIX, as well as BMAA or GLA, led to a morphological change in microglial cells, making them amoeboid (Figure 28).



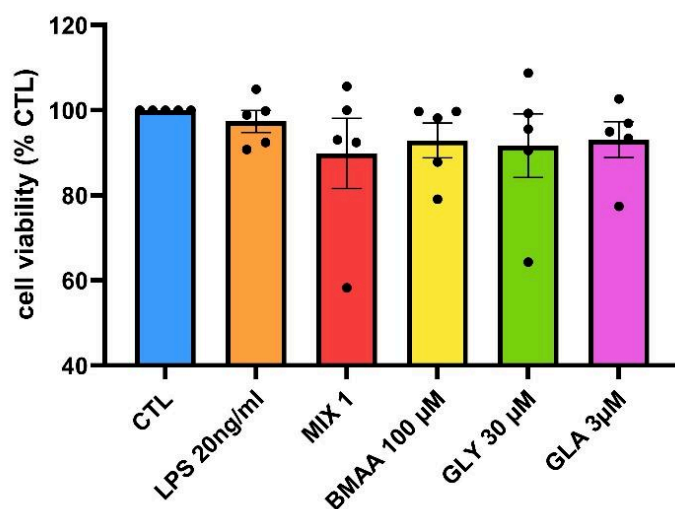
**Figure 28** – Number of IBA1-positive amoeboid cells in the whole brain of embryos exposed during the prenatal period.

These data appear to confirm the results obtained by Sarah Méresse in the context of her thesis using murine *in vitro* models of 2D and 3D cultures. She also observes a proliferation of glial cells with the MIX, but unlike the data from CEA, not with the toxins taken separately. The members of CEA also measured the protein expression of the markers CD86 and CD206,



representative of pro- and anti-inflammatory markers. The effects observed with BMAA are similar to those observed *in vitro* by Sarah Méresse, with the adoption of a pro-inflammatory status of microglial cells in embryos as well as in mothers directly exposed. These data suggest a disruption of the inflammatory response at the cerebral level.

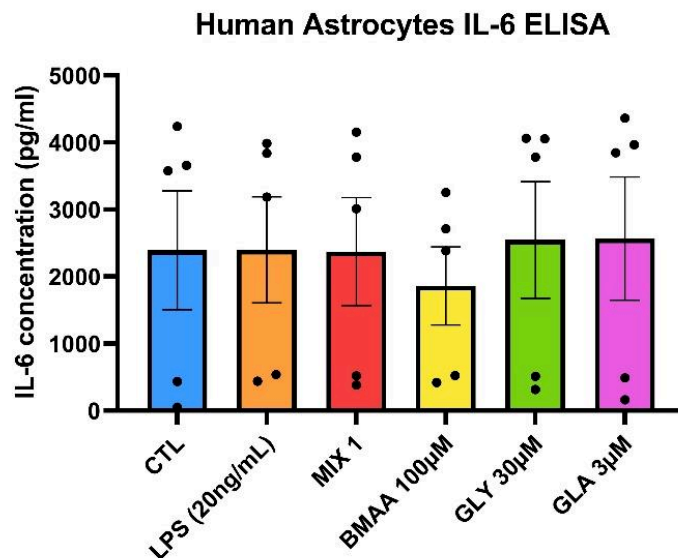
Data regarding these inflammatory processes were also investigated using an *in vitro* model of cells derived from human fetuses. Despite challenging working conditions related to the COVID-19 pandemic, I was able to obtain some results on 2D cultures of astrocytes derived from human fetuses. Indeed, the team at Macquarie University is particularly interested in neuroinflammatory processes in the etiology of neurodegenerative diseases such as motor neuron disease. By replicating the experimental protocol used in the *in vitro* model with murine cells, I evaluated the effects of repeated exposure to our compounds (alone or in a mixture) on cultures of astrocytes derived from primary human fetal cells. Contrary to what was observed in mice, I was unable to demonstrate any impact of the exposure on cell viability (Figure 29)



**Figure 29** – Measurement of cell viability measured on human fetal astrocyte cell culture.

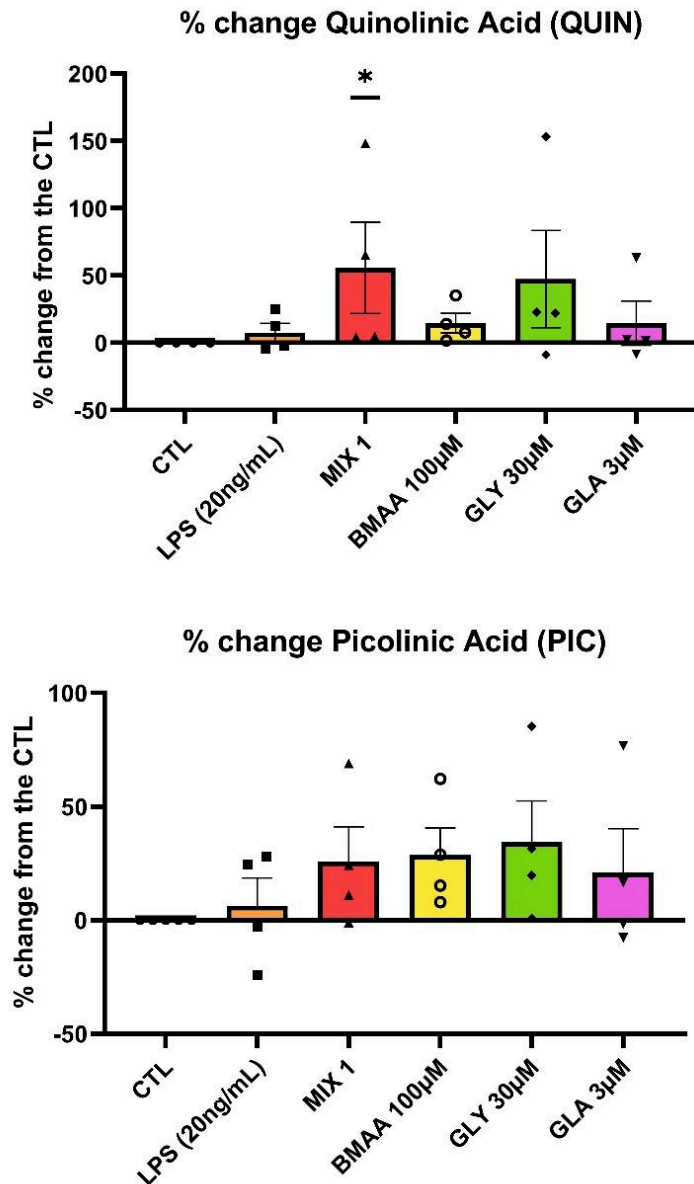
Furthermore, it appears that exposure to MIX, as well as BMAA, GLA, and GLY at the doses used, does not alter the production of pro-inflammatory cytokines such as IL-6 (Figure 30). However, after analyzing certain metabolites of the kynurenine pathway, I was able to show a significantly higher production of quinolinic acid following exposure to the mixture, an increase that is not observed with the individual molecules. As for the levels of picolinic acid, they are not affected by such exposure (Figure 31). These preliminary results are particularly interesting, especially considering that the kynurenine pathway is activated during neuroinflammation,

with a neurotoxic effect of quinolinic acid and a neuroprotective effect of picolinic acid. These initial findings lean towards a synergistic neurotoxic effect. The kynurenine pathway is the main pathway in the metabolism of tryptophan, an essential amino acid.



**Figure 30** – Measurement of the pro-inflammatory cytokine IL-6 release measured by ELISA on human fetal astrocyte cell culture.

Neuroinflammation is often characteristic of neurodegenerative diseases, such as ALS (Torbick et al., 2014). The involvement of the kynurenine pathway (KP) in ALS has been described, showing disruptions in this pathway in patients, particularly the neurotoxic effect of quinolinic acid (QUIN) (Buenz & Howe, 2007; Jaarsma et al., 2000; Karlsson et al., 2015). Deregulation of the KP is also known to be associated with various other neurological disorders such as PD (Lewis et al., 1990), multiple sclerosis (Weiss & Choi, 1988b), AD (Dunlop et al., 2013) and autism spectrum disorders (Okle et al., 2013). Already considered as biomarkers for these types of pathologies, our data suggest that studying these metabolites could allow for early identification with disruptions of this pathway and, ultimately, the possible development of associated disorders even before the appearance of symptoms. This opens up research perspectives, particularly the investigation of this pathway in our *in vivo* and *in vitro* murine models.



**Figure 31** – Fold change of quinolinic (up) and picolinic (down) acid measured by HPLC on human fetal astrocyte cell culture.

All of these observations regarding the increased sensitivity during the embryonic period directly relate to the Maternal Immune Activation (MIA) model, which highlights the importance of this period in the emergence of various disorders in offspring, occurring at different stages of life. Indeed, it is well documented in the literature that during the in utero stages, the maternal environment can affect the developing embryo. It is known that MIA is induced by infectious and non-infectious insults, serving as a significant risk factor for neuropathologies in offspring. It has been reported that increased levels of pro-inflammatory cytokines interfere with fetal brain development (Meyer, 2014; Estes et McAllister, 2016).

These often silent modifications can make individuals more susceptible to subsequent exposures, leading to phenotypic manifestations later in life (Ayhan et al., 2016). It is therefore crucial to consider this sensitive period and the associated exposures, referring directly to the concept of the "pregnancy exposome." This raises interesting questions following the events of the past three years with the COVID-19 pandemic. Speculations abound regarding the long-term effects of such an episode, particularly in pregnant women who have presented asymptomatic forms. The coming years will undoubtedly provide us initial answers regarding the potential emergence of neurodevelopmental disorders (Sureshchandra et al., 2022). Furthermore, this type of approach could also help improve our understanding of the etiology of sporadic neurodegenerative diseases.

#### **Limitations of the in vivo model in the concept of the exposome.**

However, I must still mention that replicating human pathologies is challenging. Indeed, the effects of xenobiotics can differ depending on species, doses or concentrations, as well as the route of exposure. It is known, for example, that murine metabolism is very different from that of humans, which could explain the absence of toxic effects in mice and significant effects in humans, or vice versa. Inflammatory and DNA repair systems are also species-specific. Furthermore, *in vivo* models have certain limitations, particularly in the concept of the exposome.

The exposome, referring to the totality of environmental exposures a person experiences throughout their lifetime, is fundamentally complex. It encompasses a wide range of factors, such as chemicals, physical factors, biological factors, and lifestyle. *In vivo* models cannot fully capture this complexity and are only used for specific exposures or predominant factors. Additionally, laboratory animals have shorter lifespans compared to humans, which may restrict the ability to track and understand the long-term effects of exposures on human health. Finally, the notion of inter-individual variability is challenging to account for. Each individual has undergone a unique exposome and has integrated it in a particular way due to genetic differences, lifestyle, environmental contexts, etc. *In vivo* models may not fully reflect this inter-individual variability, limiting their ability to represent the diversity of the human exposome.

However, it is important to note that despite these limitations, in vivo models remain crucial for studying the effects of environmental exposures on health and are often complementary to in vitro and epidemiological approaches in understanding the exposome and its effects.

## CONCLUSION

The aim of my thesis was to establish a multifactorial model that takes into account the several dimensions including multiple and early exposures during the perinatal period at low doses. Furthermore, the notion of inflammatory sensitization played an important role in this project, highlighting the concept of "multiple hits." Another major characteristic was the behavioral evaluation with a longitudinal monitoring. Indeed, the effects of such exposure were evaluated during early (postnatal) periods, adolescence, adulthood, and later during aging, when it was possible.

To study all these aspects, two complementary studies were conducted. The first approach explored the concept of inflammatory sensitization to determine whether an asymptomatic inflammatory episode during the gestational period sensitized the individual to a subsequent exposure to low doses of xenobiotics. The second approach, evaluated the "cocktail effect" by exposing pregnant females during the prenatal period either to a cocktail of environmental toxicants, individually or in a mixture form.

Regarding the first study, we have demonstrated that a prenatal and asymptomatic immune sensitization should be a priming effect to subsequent exposure to low doses of pollutants. This double-hits act in synergy to induce motor neurons disease related phenotypes in offspring. In the continuation of our work, we have demonstrated that maternal exposure to a low dose of xenobiotics as a cocktail, induces a synergistic, early and long lasting effects of these compounds on motor performance in offspring, which is not the case with the individual compounds.

These two studies thus demonstrate that not only the effect of multi-exposure is essential to consider, but also that the exposure period is, and particularly the gestational period, especially sensitive to the exposure of such combinations of toxicants. These findings raise an important public health issue in terms of the evaluation of the toxicological effects of various molecules, requiring further clinical, pre-clinical and epidemiological investigations taking in count

multiple-exposures to several insults into account. Therefore, it strongly emphasize to consider multiple exposures for developmental neurotoxicity, refereeing to the “maternal exposome” and pave the way for future studies aiming at deciphering cellular pathways involved in these sensitization phenomena, leading ultimately to build new adverse outcome pathways.

In spite of the limitations faced in the design of the exposome paradigm, ss models remain vital for investigating the impact of environmental exposures on health. They often complement in vitro and epidemiological approaches, contributing to a comprehensive understanding of the exposome and its effects.

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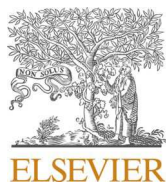
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## ANNEXES FILES

Paper Sarah Méresse:  $\beta$ -N-Methyl-Amino-L-Alanine cyanotoxin promotes modification of undifferentiated cells population and disrupts the inflammatory status in primary cultures of neural stem cells



## $\beta$ -N-Methyl-Amino-L-Alanine cyanotoxin promotes modification of undifferentiated cells population and disrupts the inflammatory status in primary cultures of neural stem cells

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One health

### ABSTRACT

$\beta$ -N-Methyl-Amino-L-Alanine (BMAA) produced by 95% of cyanobacteria is in constant augmentation with cyanobacteria worldwide proliferation due to global warming and eutrophication. Previously, it has been shown that this contaminant induced neurological disorders, notably by acting as a developmental toxin. However, very few studies focus on the impact of BMAA on neuroglial cells, like astrocytes and microglial cells, in a developmental context. In the present study, we investigated whether BMAA disturbs neurogenesis from mice sub-ventricular zone (SVZ) cells and whether this neurotoxin induces neuroinflammation. We show that BMAA at 100  $\mu$ M disturbs the population of undifferentiated cells (B1 and C cells) and promotes their proliferation. Further, BMAA affects the organization of neuroblasts, indicating that SVZ function could be impaired. BMAA affects neuroinflammatory processes by increasing the release of proinflammatory cytokines IL-1 $\beta$ , IL-6 and TNF $\alpha$ . Our study adds to evidence that BMAA may disturb the central nervous system homeostasis by targeting glial cells. We highlighted that BMAA may impair SVZ niches and drives astrocytes and microglial cells into a proinflammatory status, with an amoeboid shape for microglia.

### 1. Introduction

The involvement of environmental pollutants in the development of nervous system pathologies has been proposed for several years. The  $\beta$ -N-Methyl-Amino-L-Alanine cyanotoxin (BMAA) is a non-proteinaceous amino-acid produced by two distinct groups of microalgae, diatoms and dinoflagellates (Jiang et al., 2014; Lage et al., 2014), and also by more than 95% of all cyanobacterial species (Cox et al., 2005). It is suspected of causing neurodegenerative disorders. Indeed, this toxin is increasingly considered a potential environmental risk factors for sporadic neurodegenerative diseases outcome, such as Alzheimer's Disease (AD), amyotrophic lateral sclerosis and amyotrophic

lateral sclerosis/parkinsonism-dementia complex (Caller et al., 2018; Cox et al., 2016, 2018; Pablo et al., 2009).

Cyanobacteria are present worldwide especially in aquatic environments. Global warming and eutrophication both contribute to increasing algae blooms and consequently the presence of BMAA (Paerl and Huisman, 2008). Thus, BMAA represents a global public health issue due to the chronic exposition of humans and other species. Recreational waters and seafood are the main sources of contamination (Réveillon et al., 2015). Indeed, this toxin has been detected especially in filter-feeding bivalves and fish (Masseret et al., 2013; Réveillon et al., 2014; Jiang et al., 2014; Lance et al., 2018). BMAA can bioaccumulate among the trophic chain (Cox et al., 2003) leading to high amounts in

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apex predators. Recently, studies of the marine environment have shown high levels of BMAA in sharks and dolphins, with neurodegenerative alterations in the latter (Mondo et al., 2012; Hammerschlag et al., 2016). Disturbingly, BMAA was also measured in the human central nervous system (CNS) (Pablo et al., 2009; Berntzon et al., 2015). Current evidence suggests that neurodegenerative diseases have multifactorial and complex origins but also that they have their roots early in the developmental period (Tartaglione et al., 2015). Indeed, the neurodevelopmental process is a critical and particularly sensitive period to exposure to toxicants. A disturbance during this period may therefore alter the development of neural structures and/or lead to short- and long-term adverse consequences (Rice and Barone, 2000; Padmanabhan et al., 2016). The core principle is the basis for the Barker hypothesis known as the Developmental Origin of Health and Disease (DOHaD) approach (Sinclair and Singh, 2007).

Therefore, neural stem cells (NSCs) appear clearly as a useful *in vitro* model to study the mechanisms of developmental neurotoxicology (Tofghi et al., 2011). Indeed, NSCs have the potential to differentiate into neurons, oligodendrocytes, astrocytes and to self-renew sufficiently to generate the CNS from a reduced number of NSCs (McKay, 1997; Kriegstein and Alvarez-Buylla, 2009). Two regions of the mature mammalian brain generate new neurons: the subgranular zone of the dentate gyrus of the hippocampus and the subventricular zone (SVZ). The process of neurogenesis starts with B1 cells (Bonaguidi et al., 2011; Merkle et al., 2004) form transient amplifying type C cells by asymmetric cell division. Type C cells in turn form neuronal progenitor cells (neuroblasts, type A cells) astrocytes. Recently, Pierozan et al. (2020) showed that hippocampal neural stem cells are more susceptible to BMAA than primary neurons. They demonstrated that BMAA induced apoptosis in NSCs at a dose twelve times lower than that required to decrease viability in primary neuron cultures. In addition, low BMAA concentration inhibited neural stem cells differentiation into neurons, astrocytes, and oligodendrocytes. However, while some studies highlighted the potential neurotoxic effects of BMAA on these neurodevelopmental cells (Scott and Downing, 2017; Pierozan and Karlsson, 2019) the effect of BMAA on the neuroinflammatory function, especially mediated by astrocytes and microglia remained to be characterized (Stephenson et al., 2018).

In this context microglial cells, considered the resident macrophage of the CNS are particularly important to consider. Indeed, it has been demonstrated that microglia has crucial and distinct roles during development, including regulating cell differentiation and neurogenesis. Microglia phagocytose several cell products from dead, dying, and living cells (Lenz and Nelson, 2018; Cunningham et al., 2013) and secrete a set of cytokines such as IL-1 $\beta$  and TNF $\alpha$  modulating the neurodevelopmental events (Butovsky et al., 2006; Shigemoto-Mogami et al., 2014).

Therefore, in the current study, we investigated the impact of chronic exposure to low BMAA concentration according to the literature during cellular differentiation of NSCs primary culture from SVZ containing microglia. After assessing BMAA-induced cytotoxicity and viability, we characterized the cell population after one week of differentiation. Further, in order to elucidate whether BMAA induces neuroinflammation, we investigated the activation status of the immunocompetent cells, astrocytes and microglial cells, the morphological changes of microglial cells and the release of proinflammatory cytokines induced by BMAA.

## 2. Material and methods

### 2.1. Animals and animal care

C57BL/6JRj breeding mice (Janvier Labs, Le Genest-St-Isle, France) were housed in a temperature and humidity-controlled environment on a 12 h light/dark cycle with ad libitum access to water and food. Animal care and experimentation were in accordance with the European

Communities Council directive (2010/63/EU) and the French Minister under APAFIS#21860.

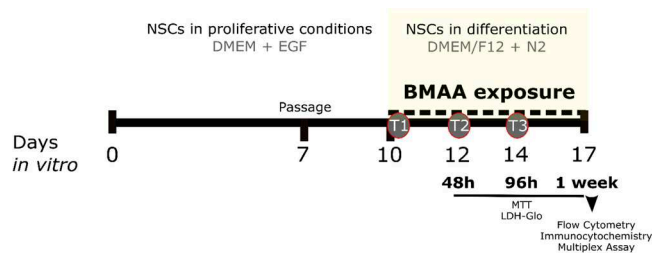
### 2.2. Cell culture and BMAA exposure

*In vitro* murine primary NSCs cultures from SVZ neurogenic niches were performed on postnatal days 1–5 mice and NSCs were cultured as previously described (Delgehyr et al., 2015; Feat-Vetel et al., 2018). NSCs culture was made from at least four pups. Briefly, to obtain a single-cell suspension the olfactory bulbs and hippocampus were removed from neonatal brains and the meninges were peeled off. The dissected telencephalon was mechanically dissociated before enzymatic digestion with papain (Sigma, P4792). The cells were then resuspended in DMEM medium (Sigma, D5671), completed with 10% Fetal Bovine Serum (FBS, GE Healthcare HyClone), 1000 units/mL Penicillin-Streptomycin (GIBCO 15070–063) and 2 mM of L-Glutamine (GIBCO, 25030–024). The cells were plated on poly-L-lysine (5  $\mu$ g/mL, BD Biosciences) precoated 25 cm<sup>2</sup> flasks. Two flasks were used for one brain. NSCs are maintained in cultures at 37 °C with 5% CO<sub>2</sub> in a humidified incubator until confluence. To avoid toxicity of cellular debris, medium was replaced the following day, and then every second day, with the medium containing Epidermal Growth Factor (EGF, 30 ng/mL Sigma E9644) to keep cells in an undifferentiated status. When the cells reach confluence, usually 7 days after plating, they were detached with trypsin (Sigma, 59417 C) and plated at  $1.5 \times 10^5$  cells/well, in the presence of EGF. Five days after passaging, the medium culture was replaced by DMEM/F12 (Gibco, 11320033) completed with N-2 supplement (Fisher Scientific, 11520536) to promote differentiation into neuroglial cells. Cells were treated every second day for one week with concentrations of 100  $\mu$ M to 3 mM BMAA (CAS number 16012–55–8,  $\geq$  97%, Sigma, B107 during differentiation as indicated (Fig. 1).

### 2.3. Cytotoxicity and cell viability

#### 2.3.1. LDH assay

Lactate dehydrogenase (LDH) is a soluble and stable cytosolic enzyme present in many cell types that is rapidly released into the cell culture medium upon disruption of the plasma membrane. LDH release was measured using the LDH-Glo™ Cytotoxicity assay (Promega, J2380) following the manufacturer's protocol. Briefly, 5  $\mu$ l of supernatant medium was collected from each sample after 48 h, 96 h and one week of treatment and stored at – 20 °C into the storage buffer. LDH levels are measured by adding an equal volume of LDH Detection Reagent to the



**Fig. 1.** Experimental protocol for the evaluation of the effects of BMAA in NSCs. *In vitro* murine primary NSCs were isolated from SVZ at day 0. After 7 days under proliferative conditions, cells were passaged and maintained in the same medium. The cells were then treated every second day with BMAA at the same time as medium change, at different concentrations (100  $\mu$ M, 300  $\mu$ M and 3 mM) while the differentiation occurs. 48 h, 96 h and one week after the first treatment the viability and the cytotoxicity were assessed by MTT and LDH-Glo assay. After one week of treatment, corresponding to a total of three treatments, cells populations and activation status were define by flow cytometry. At the same time-point the proliferation of undifferentiated cells, the population of neuroblast, and the morphology of microglial cells were assessed by immunocytochemistry. The release of proinflammatory cytokines was measured by Multiplex assay.



diluted sample. The luminescence was recorded after 40 min using a microplate reader (ClarioStar, BMG LABTECH).

### 2.3.2. MTT assay

Cell viability was measured by the 3-(4,5-dimethyl-2-yl)2,2,5-diphenyl-2-H-tetrazolium bromide (MTT, Sigma, M2003) assay. This test is based on the conversion of the tetrazolium salt to the colored product formazan by succinate dehydrogenase, which is a mitochondrial enzyme. Viability was measured after 48 h, 96 h and one week of treatment, in the primary culture of neural stem cells in differentiation. Briefly, NSC were plated at  $4 \times 10^4$  cells per well in 96-well plates. At the end of exposure, MTT was added to the medium (5 mg/mL) and incubated for three hours. The formazan product generated was solubilized in dimethyl sulfoxide for 15 min and measured at 570 nm using a microplate reader (ClarioStar, BMG LABTECH).

### 2.4. Immunocytochemistry

To study cellular proliferation and differentiation, immunocytochemistry staining method was performed. Briefly, NSCs were plated at  $1 \times 10^5$  cells per well in 24-well plates containing Poly-L-Lysine (Sigma, P1524) precoated glass coverslips. After one week of exposure, the medium was removed and the coverslips were washed with Tris-Buffer Saline 1X (TBS, pH 7.6, Tris-HCl 50 mM, NaCl 150 mM), fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. PFA was then washed with TBS. Cells were then permeabilized with TBS containing 10% FBS; 0,2% Triton X-100 (Euromedex, ref 2000-A) and 2% Bovine Serum Albumin (BSA, Sigma, A9418) for 30 min at room temperature. Coverslips were then incubated at least three hours at room temperature using the following antibodies: anti-Ki67 (1:200, polyclonal IgG rabbit, Abcam ab15580); anti-Nestin (1:500, monoclonal IgG1k mouse, Abcam ab11306); anti-Doublecortin (1:500, polyclonal IgG rabbit, Abcam ab77450) for neuroblasts (A cells); anti-Iba1 (1:250, polyclonal IgG goat). Coverslips were rinsed with TBS and incubated with appropriate secondary antibodies (1:500, anti-rabbit, anti-mouse or anti-goat Alexa 488 or TRITC-conjugated) for 45 min, also at room temperature in TBS solution with 10% FBS solution and 2% BSA. Then, coverslips were rinsed and incubated two minutes with DAPI, to visualize the cell nuclei. Coverslips were rinsed three times in TBS and once in distilled water before being mounted on glass slides in mounting-medium Fluoromount-G (Clinisciences K024–200). Microscopic images were obtained using a fluorescent microscope Leica DM6000B with Metamorph software with 20x or immersed 100x magnification. For each condition, at least three replicates were studied. For each replicate, at least three fields were scored per coverslip and then averaged. Distances and dimensions were measured using the Image J® software (NIH Image, Schneider et al., 2012).

### 2.5. Flow cytometry

Cellular populations, differentiation and neuroinflammatory status were assessed by flow cytometry. NSCs were plated at  $1 \times 10^5$  cells per well in 6-well plates. After one week of exposure, cells were stained with fluorescent label antibodies: Fixable Viability Dye (eBiosciences™, 65–0865–14, 1/800), anti-ACSA2 APC (MILTENYI, 130–117–386, 1:50), anti-CD45 V450 (BD Horizon™, 560501, 1:100), anti-CD11b PerCP/Cy5 (BD Pharmingen™, 560993, 1:100), anti-CD86 FITC (BD Biosciences, 561961, 1:100), anti-CD206 PE-Cy7 (BioLegend, 141720, 1:100). Non-conjugated anti-CD16/32 antibody (BD Pharmingen™, 553142, 1:100) was used to block non-specific Fc receptors for 20 min at 4 °C. Then, the cells were washed and before fixation, neural stem cells population were stained with intracellular Nestin PE (MILTENYI, 130–119–799, 1:50), after cell permeabilization for 20 min at 4 °C with Cytofix/Cytoperm Plus Kit (BD Biosciences, Paris, France). Cells were then washed and resuspended in lysing solution (BD FACSTM Lysing Solution) before the acquisition. Data were acquired with a flow

cytometer (BD FACSCanto II) and analyzed with FlowJo v10 software (Tree Star, Ashland, OR). Very low SSC and very low FSC were excluded to strictly define the populations of interest. For the analysis, single live cells were pre-gated. Activation status was defined using the ratio CD86/CD206 obtained after staining with anti-CD86 antibody for pro-inflammatory status and anti-CD206 antibody for anti-inflammatory status.

### 2.6. MULTIPLEX assay

Supernatant samples were collected after one-week of exposure and differentiation of NSCs culture. A positive control was made with LPS at 100 ng/mL during four hours of treatment. Samples were stored at  $-80$  °C until analyzed. All measurements were obtained from custom ProcartaPlex™ panels created by combining multiple simplex bead sets. IL-1 $\beta$ , IL-6 and TNF $\alpha$  concentrations were determined according to the manufacturer's instructions using multiplex assay (Thermo Fisher, PPX-06).

### 2.7. Statistics

The results are presented as mean  $\pm$  SEM for each experimental group. Each point corresponds to a biological replicate. Since our data did not follow a normal distribution, differences compared with the control were analyzed by Mann-Whitney or Kruskal-Wallis tests according to the number of groups, as non-parametric data. Using GraphPad Prism 9® (Graphpad Software, San Diego, CA, USA). *P* values  $\leq 0.05$  were considered as statistically significant.

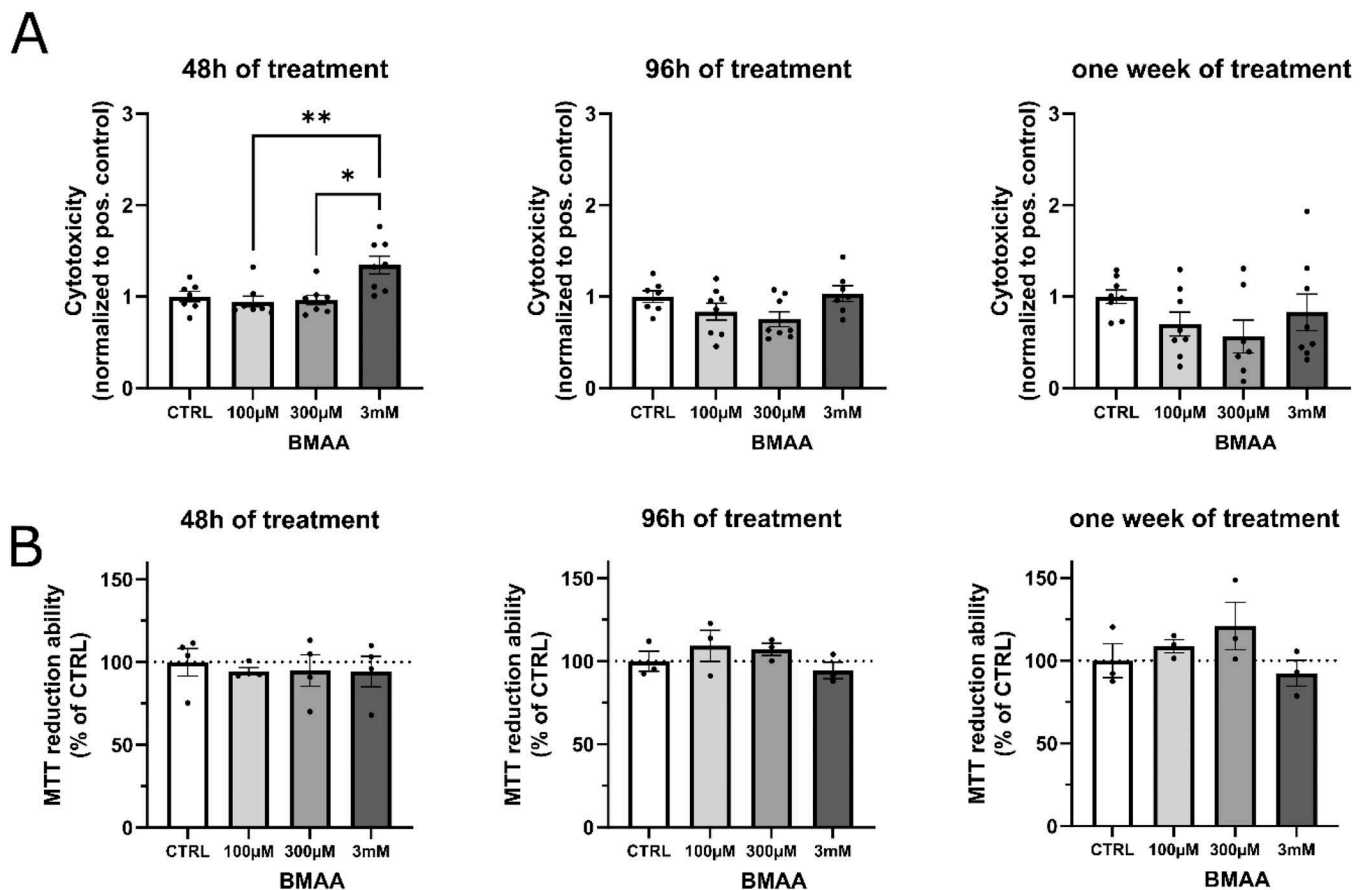
## 3. Results

### 3.1. Limited effects of BMAA exposure on cell toxicity and cell viability

We first aimed to determine if BMAA induces cytotoxicity or disturbs viability at 100  $\mu$ M, 300  $\mu$ M and 3 mM concentrations in neural stem cells. This was evaluated after 48 h, 96 h and one week of treatment during differentiation into neuroglial cells according to our experimental design (Fig. 1). The highest, 3 mM BMAA concentrations induced cytotoxicity after 48 h of treatment at 3 mM as compared to lower concentrations. This effect was transient or reversed 96 h or one week of treatment. After one week of treatment there seemed to be less LDH release in the medium, and therefore cytotoxicity (not significant) (Fig. 2A). Whatever the exposure time, BMAA exposure did not affect the viability (Fig. 2B). The highest concentration of BMAA studied was devoid of cell toxicity was 300  $\mu$ M. The concentration of 100  $\mu$ M was chosen to further analyze the effects of BMAA on cell differentiation, cell populations, and neuroinflammatory processes. This concentration is considered low in the literature.

### 3.2. BMAA exposure favors neural stem cells proliferation ability and impairs differentiation of undifferentiated cells

The effect of BMAA on proliferation of undifferentiated cells was studied by immunocytochemistry using Ki67 as a marker of proliferation and the maintenance of proliferation ability. To discriminate neural stem cells a Nestin staining was performed. Particular attention was paid to double-labeled cells. Representative images of the experiment are shown (Fig. 3A). The growth based on the total cell number was not affected by 100  $\mu$ M of BMAA at the end of the differentiation phases. Moreover, there was no modification of the proportion of cells in proliferation in cell culture. Thus, BMAA affected neither cell growth nor proliferation under these conditions (Fig. 3B). Interestingly, the proportion of Nestin<sup>+</sup> undifferentiated neural cells in ability to proliferate was significantly higher after BMAA treatment. This suggested an imbalance in the population of undifferentiated cells which are B1 cells and C cells (Fig. 4A). Our in vitro cell culture model represents the



**Fig. 2.** Effects of BMAA on cell toxicity and viability in neural stem cells (NSCs) culture. Cell toxicity was determined using the LDH-Glo assay after treatment with 100 µM to 3 mM BMAA for 48 h, 96 h and one week during differentiation; 10% Triton X-100 was used as positive control. For 48 h of treatment CTRL n = 7; BMAA 100 µM n = 7; 300 µM n = 7; 3 mM n = 8. 96 h of treatment CTRL n = 7; BMAA 100 µM n = 8; 300 µM n = 8; 3 mM n = 7. One week of treatment CTRL n = 8; BMAA 100 µM n = 8; 300 µM n = 7; 3 mM n = 7 (A). Cell viability was determined using the MTT assay after treatment with 100 µM to 3 mM BMAA for 48 h, 96 h and one week during differentiation. For 48 h of treatment CTRL n = 4; BMAA 100 µM n = 3; 300 µM n = 4; 3 mM n = 4. 96 h of treatment CTRL n = 3; BMAA 100 µM n = 3; 300 µM n = 3; 3 mM n = 3. One week of treatment CTRL n = 3; BMAA 100 µM n = 3; 300 µM n = 3; 3 mM n = 3 (B). Values are normalized and represent mean ± SEM. Statistical differences are indicated: \*p < 0.05, \*\*p < 0.01 using Kruskal-Wallis test.

different stages of formation of several cell types characteristic of the SVZ (Feat-Vetel et al., 2018). Thus, we characterized the population of undifferentiated cells in detail by flow cytometry. A scheme of the specific staining used to discriminate each cellular population is shown (Fig. 4A) and representative dot blots are shown (Fig. 4B). Here, B1 cells were double-stained by Nestin and ACSA2 (astrocyte cell surface antigen-2). This antibody has been developed for the detection of astrocytes and also stain B1 cells (Kantzer et al., 2017). C cells were stained only by Nestin and astrocytes were only stained by ACSA2. Exposure to BMAA led to a small but significant decrease of B1 cell population, correlating with a trend increase of C cell populations (Fig. 4C). We did not observe changes in astrocyte populations.

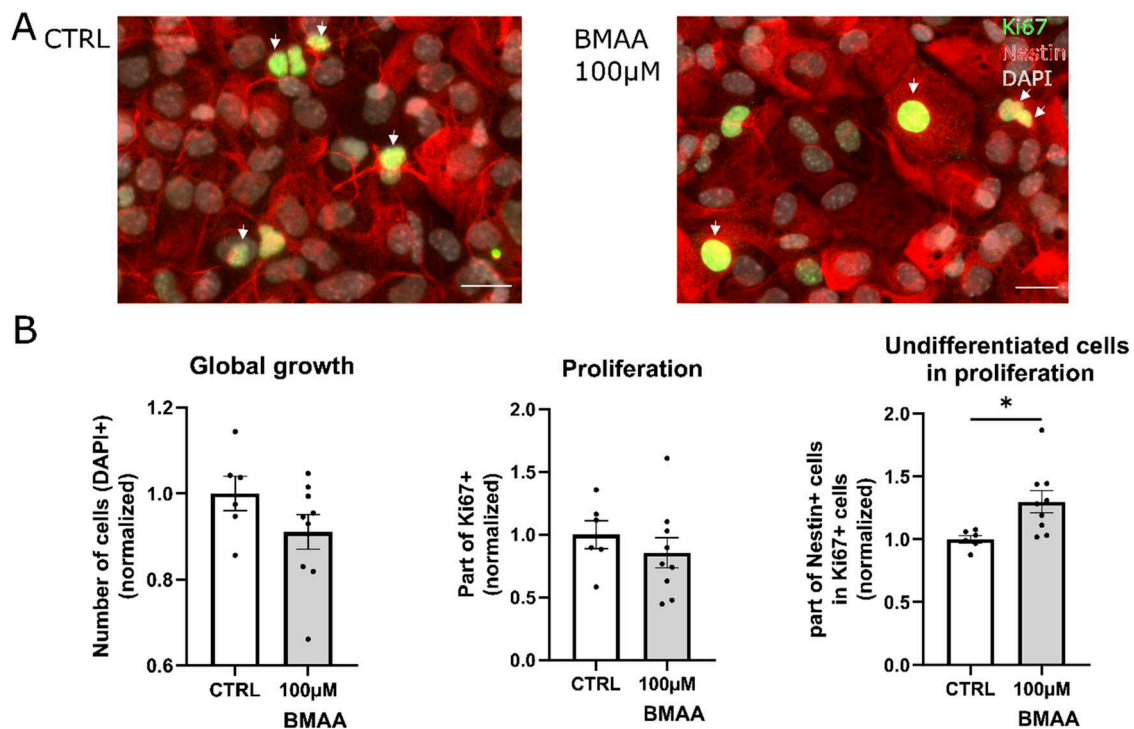
### 3.3. BMAA disturbs the organization of neuroblasts

One of the characteristics of this cell culture model is also its ability to generate neuroblasts, like what occurs in SVZ. Similar to the *in vivo* situation, these migrating cells come together in chains and clusters. We demonstrated previously that environmental pollutants could affect the SVZ neuroblast population (Feat-Vetel et al., 2018). Thus, we next evaluated the effect of BMAA on the neuroblast population and organization by immunocytochemistry. To study the organization of neuroblasts we considered that two contiguous cells constituted a group and a scattered neuroblast is a neuroblast that was not in a cluster. Specific staining of neuroblasts by doublecortin (DCX) antibody is shown

(Fig. 5A). Cell growth based on the total cell number was not affected by 100 µM of BMAA at the end of the differentiation phase and neither was the number of neuroblasts (Fig. 5B). While the number of clustered and scattered neuroblasts were not affected, and the number of DCX<sup>+</sup> cell clusters significantly increased after differentiation in presence of BMAA indicating that the organization of neuroblasts was affected (Fig. 5C).

### 3.4. Effects of BMAA exposure on the inflammatory status of B1 cells and astrocytes

NSCs may contribute to moderating neuroinflammation, mainly induced by immunocompetent cells such as astrocytes and microglia. Thus, we next assessed the effect of BMAA on the inflammatory status of B1 cells and astrocytes by flow cytometry. ACSA2 staining was used to label both B1 and astrocyte cell types; we were not able to distinguish those cell types. CD86 as a costimulatory marker of proinflammatory status and mannose receptor CD206 as marker of anti-inflammatory cells. Representative images are shown (Fig. 6B). BMAA didn't affect the population of B1 cells and astrocytes (Fig. 6C). Interestingly, ACSA2<sup>+</sup> cells showed higher expression of CD86, the surface phenotype marker of proinflammatory status and less expression of CD206 the marker of anti-inflammatory status. To illustrate the balance between proinflammatory and anti-inflammatory status, we represented the ratio of CD86/CD206. It was significantly increased after one week of treatment with BMAA at 100 µM suggesting that ACSA2<sup>+</sup> cells were under a



**Fig. 3.** Proliferation of undifferentiated cells after exposition to BMAA in neural stem cells (NSCs) culture. Cells proliferation was determined by immunocytochemistry with Nestin staining to discriminate undifferentiated cells and Ki67 for proliferative cells; Scale bar = 20  $\mu\text{m}$  (A). Quantification of the total number of cells per field, proportion of proliferative cells and the proportion of Nestin positive cells in the proliferative cells populations after BMAA (100  $\mu\text{M}$ ) treatment as compared to untreated controls (B). Values represent mean  $\pm$  SEM, normalized (CTRL n = 6; BMAA 100  $\mu\text{M}$  n = 9). Statistical differences from control are indicated: \*  $p < 0.05$  using Mann-Whitney test.

proinflammatory status (Fig. 6C).

### 3.5. Effects of BMAA exposure on inflammatory status of microglial cells

Our in vitro primary culture model also has the advantage of containing a population of microglial cells. These cells are particularly important for neuroinflammation but also for SVZ homeostasis (Colonna and Butovsky, 2017). Indeed microglial cells have a key role in neural stem cell differentiation (Butovsky et al., 2006; Shigemoto-Mogami et al., 2014). Considering this, we next assessed the effect of BMAA on the inflammatory status of microglial cells by flow cytometry. CD11b and CD45 staining was used to define microglial cells and CD86 and CD206 were used to determine their activation status. Representative images are shown (Fig. 6D). BMAA treatment did not affect the frequency of microglial cells in SVZ NSCs culture (Fig. 6E). Microglial cells also showed higher expression of CD86 and less expression of CD206; the ratio of CD86/CD206 was drastically increased after one week of treatment with BMAA at 100  $\mu\text{M}$ , suggesting that microglial cells were also under a proinflammatory status (Fig. 6E).

### 3.6. BMAA turns microglial cells into amoeboid shape

Based on the suggested proinflammatory status of BMAA-exposed microglial cells, the morphology of these cells were studied by immunocytochemistry. Representative images of Iba1<sup>+</sup> microglial cells are shown (Fig. 7A). Indeed, Zanier et al. (2015) showed that the activated microglia could be characterized by their shape especially in terms of solidity and circularity. Here we show that BMAA exposure caused morphological modifications of microglial cells. Indeed, the results revealed a decrease of more than 20% of the area and the perimeter compared to control, indicating that microglia cells were smaller in BMAA conditions, as compared to controls (Fig. 7B). The significant increase in both solidity and circularity parameters indicated a larger

proportion of microglia in amoeboid shape after BMAA exposure as compared to controls. Thus, BMAA exposed microglia exhibit morphological characteristics of proinflammatory activated status.

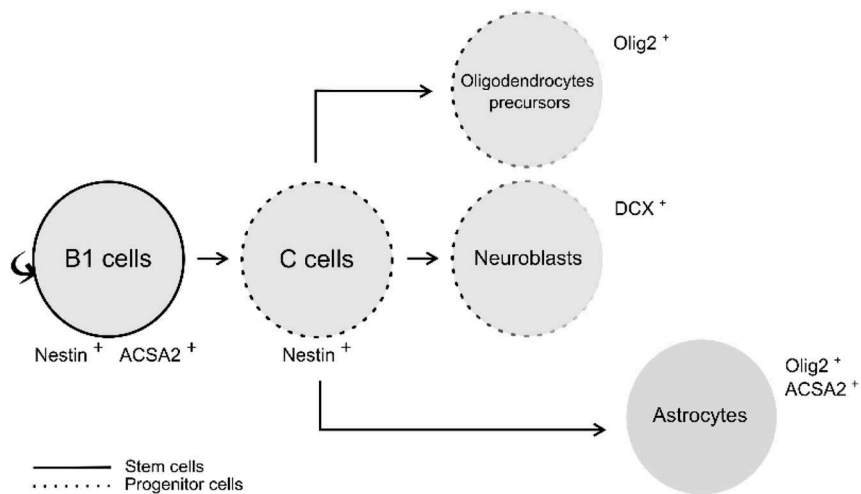
### 3.7. BMAA exposure triggers the release of proinflammatory cytokines

Our cell culture model derived from SVZ NSCs allows us to study the interactions between cell populations, such as astrocytes and microglia, which can lead to the release of neuroinflammation mediators. When microglia and astrocytes were in proinflammatory status we evaluated the effect of BMAA on proinflammatory cytokine release, such as IL-1 $\beta$ , IL-6 and TNF $\alpha$ , which are considered major mediators of neuroinflammation. Multiplex analysis showed that exposure to BMAA at 100  $\mu\text{M}$  for one week, induced an increase of IL-1 $\beta$ , IL-6 and TNF $\alpha$  release in the supernatant (Fig. 8). For each cytokine, the increase was about twofold. Thus, BMAA exposure triggered a sustained release of proinflammatory cytokines in SVZ NSCs culture.

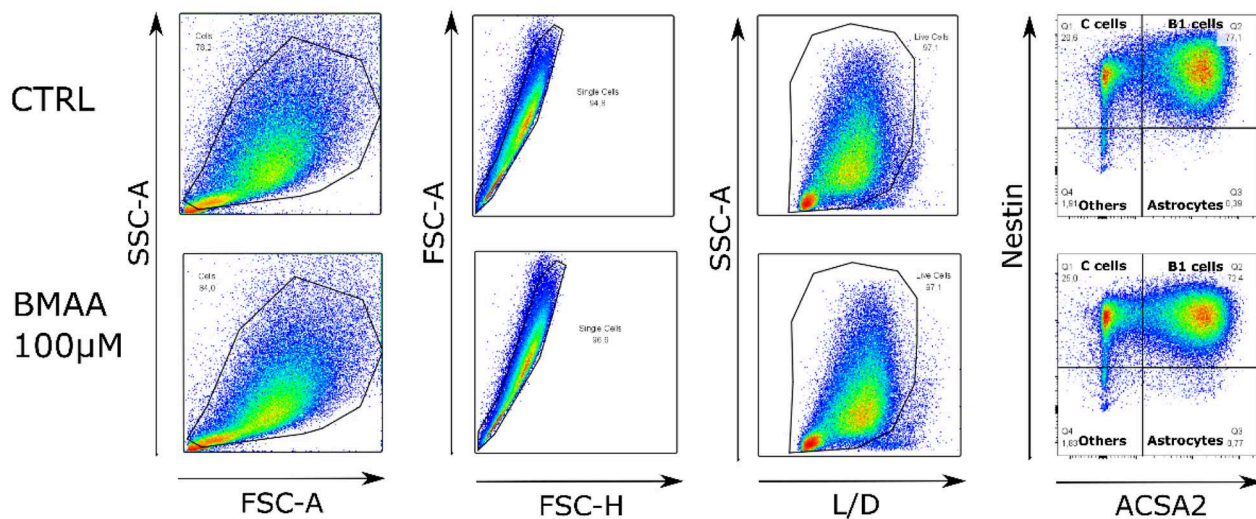
## 4. Discussion

The central nervous system development requires an intricately orchestrated balance between the proliferation of the multipotent neural stem cells, differentiation into the appropriate cell types, and the subsequent migration of these cells. During the development of the CNS, cell proliferation, migration and differentiation are highly sensitive to environmental exposure (Coullery et al., 2016). Slight perturbation of these neurodevelopmental processes may lead to impairments of the brain and could have long-term effects on adult neuropathologies (Homem et al., 2015). Here, we established an in vitro neurotoxicity assay based on neonate SVZ. Our culture model allows us to evaluate the impact of toxins such as BMAA on the early stages of neurogenesis. In addition, we can assess the role of early toxin exposure on neuropathies involving an environmental component. Due to the presence of both

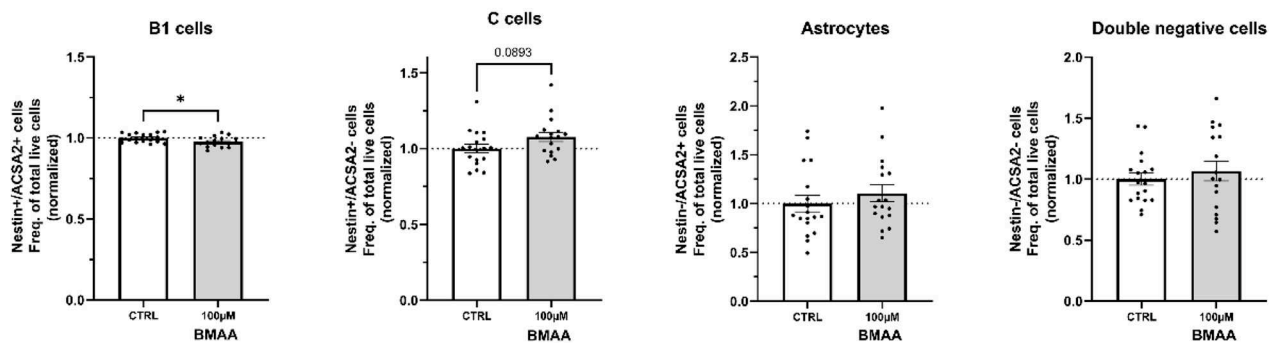
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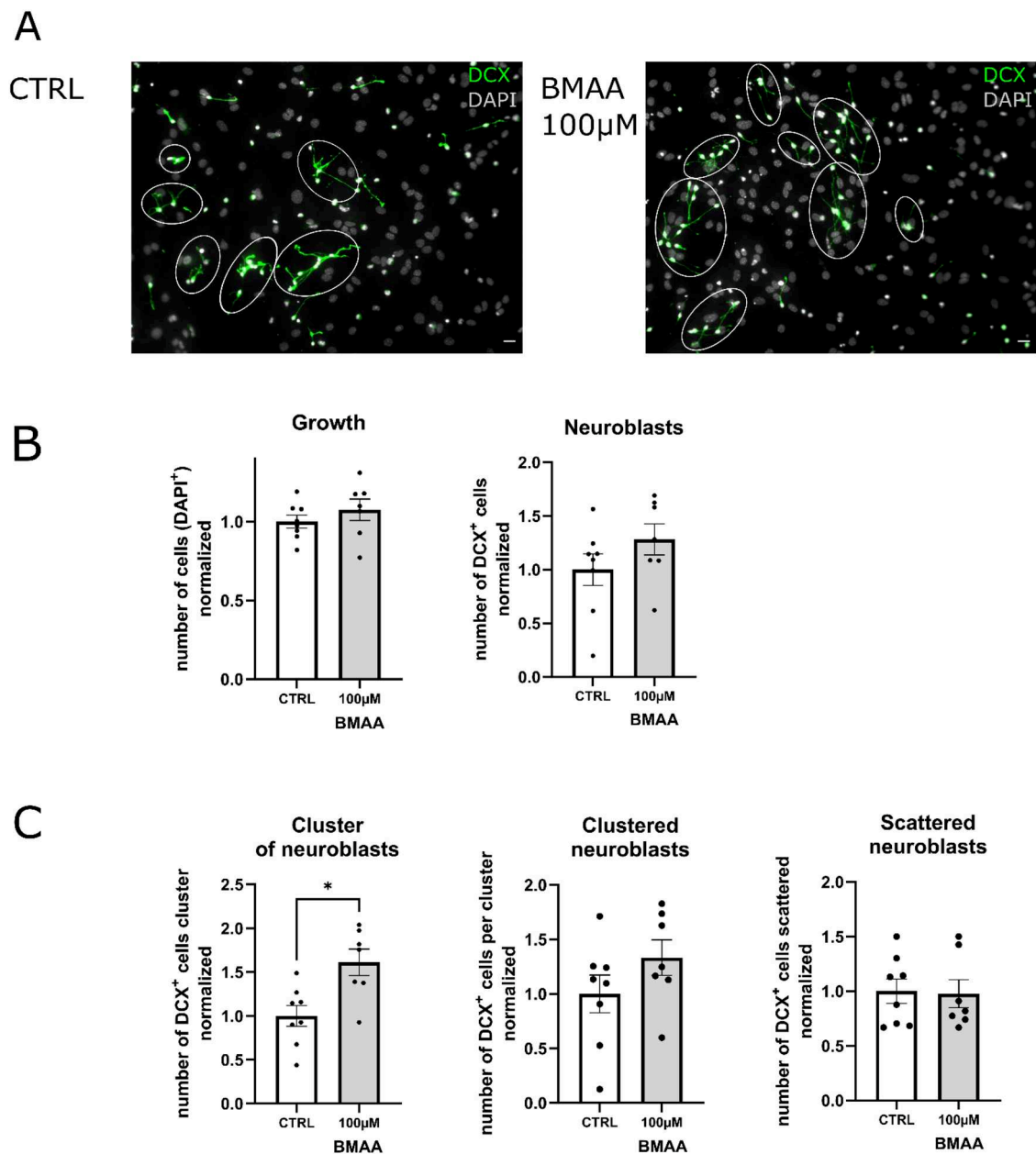
B



C



**Fig. 4.** Effects of BMAA on neural stem cells (NSCs) differentiation. Cells populations were determined by flow cytometry; Staining to determine cell populations of B1, C and Astrocytes cells (A). A gate was created on non-debris populations, excluding very low SSC/FSC events. Cells were gated on single cells and selected by Live/Dead staining. Then, populations were gated according to Nestin and ACSA2 staining (B). Quantification of B1 cells, C cells and Astrocytes and other cells which are negative to both staining after BMAA (100 µM) treatment as compared to untreated controls (C). Values represent mean ± SEM, normalized (CTRL n = 18; BMAA 100 µM n = 17). Statistical differences from control are indicated: \* p < 0.05 using Mann-Whitney test.

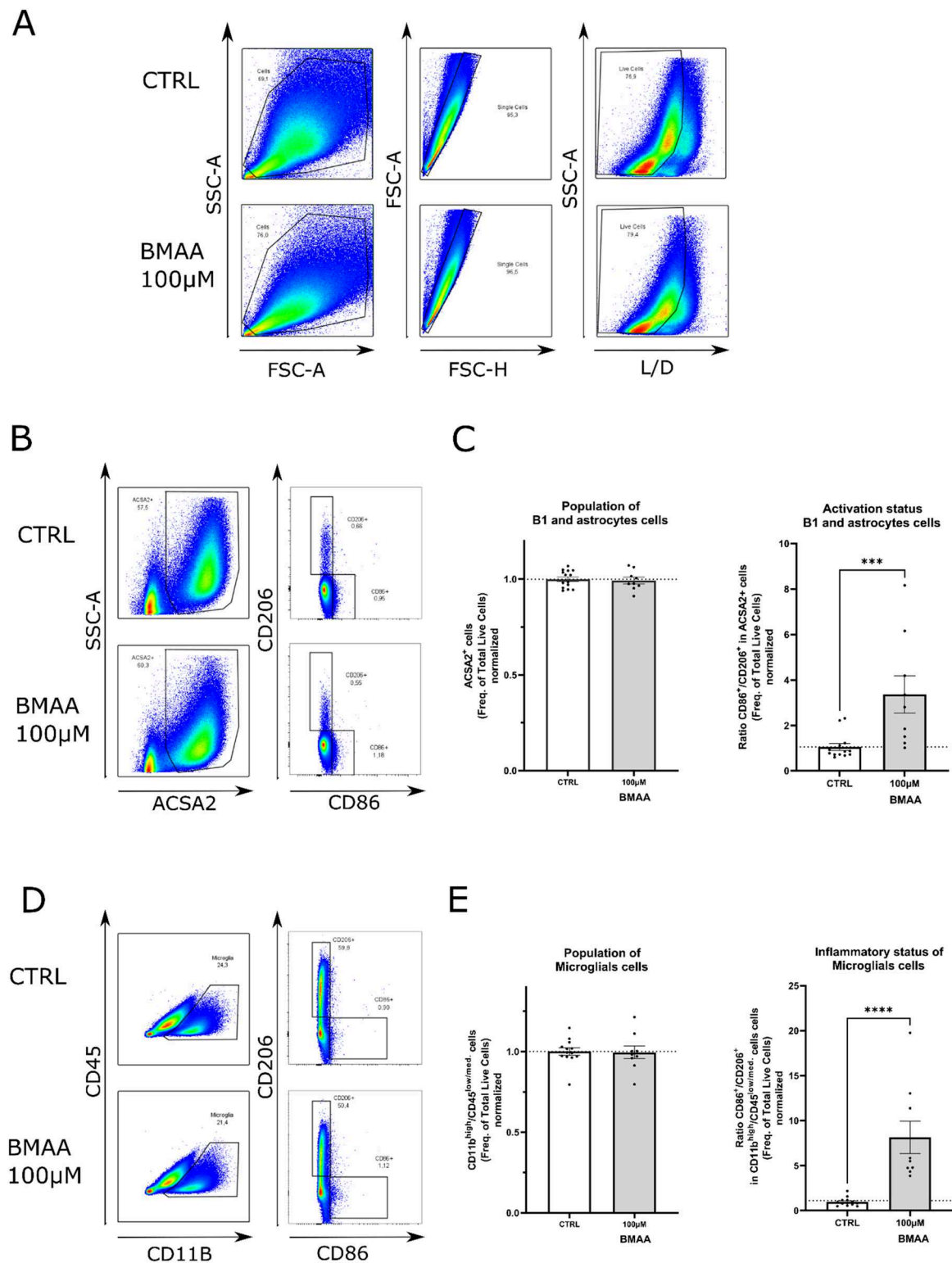


**Fig. 5.** Effects of 100 µM of BMAA on population and organization of neuroblasts from neural stem cells (NSCs). Neuroblasts were determined by immunocytochemistry using DCX staining; scale bar = 20 µm (A). Quantification of the total number of cells per field to illustrate the growth and the percentage of DCX positive cells (B). Quantification of the organization of neuroblasts; number of cluster, number of neuroblasts per cluster and number of scattered neuroblasts after BMAA (100 µM) treatment as compared to untreated controls (C). Values represent mean ± SEM (CTRL n = 8; BMAA 100 µM n = 7). Statistical differences are indicated: \*p < 0.05 using Mann-Whitney test.

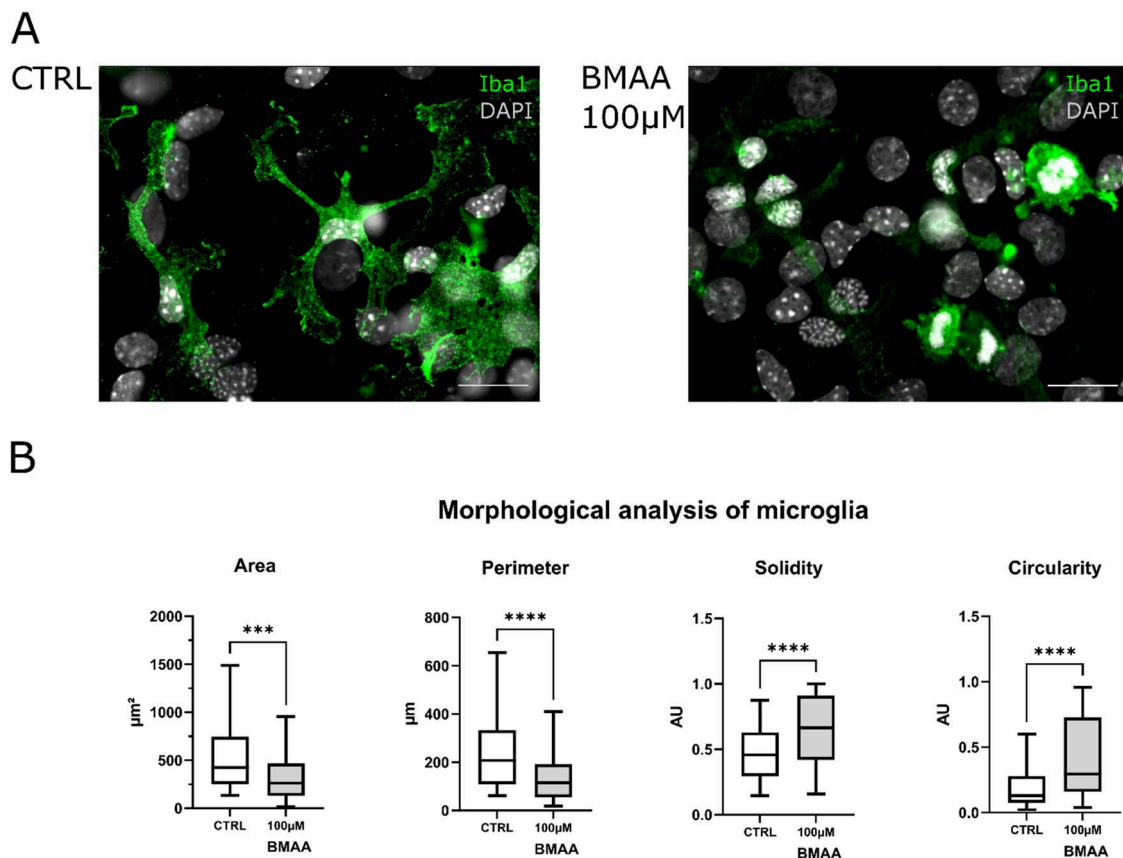
immunocompetent cells, astrocytes and microglial cells, we are also able to assess neuroinflammation.

We showed that BMAA toxin enhances the proliferative ability of undifferentiated cells originating from SVZ derived NSCs culture after one week exposure. This was accompanied by a decrease in B1 cells and an increase in C cell population. Many pollutants are already known to have adverse effects on glial differentiation from SVZ niches (Masood et al., 2021; Tiwari et al., 2015; Wang et al., 2017). In the nervous system, keeping a certain proportion of each population is a prerequisite for maintaining its normal structure and function. SVZ harbors the NSCs and represents the main site of primary and secondary neurogenesis (Inta et al., 2008; Lim and Alvarez-Buylla, 2016). On the other neurogenic niche, exposure to 100 µM of BMAA inhibited the differentiation of NSCs into astrocytes, neurons and oligodendrocytes in the dentate

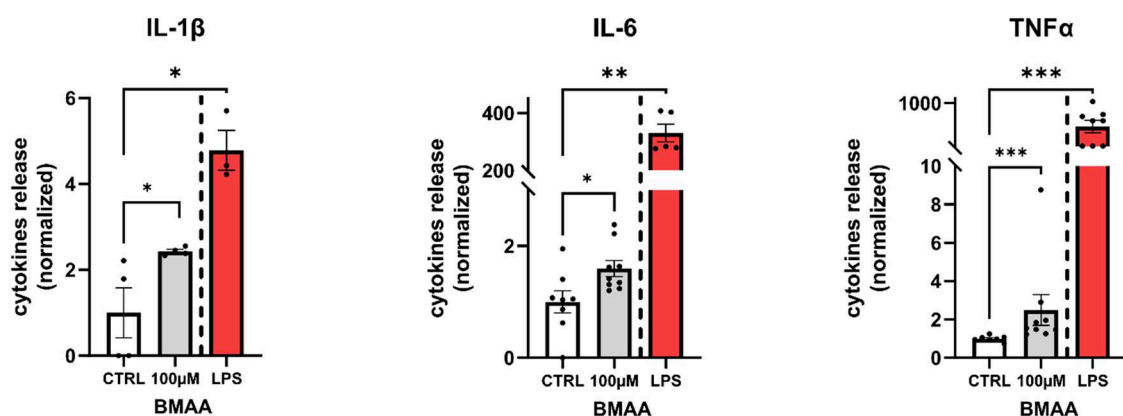
gyrus of hippocampus (Pieroza et al., 2020). Our study further adds evidence to previous research indicating that the effects of BMAA can differ depending on the brain area and therefore cells type (Pieroza and Karlsson, 2019). Indeed, Pieroza and Karlsson reported that exposure to BMAA reduced striatal neural stem cells proliferation (Pieroza and Karlsson, 2019) and they also showed an increase in the percentage of undifferentiated cells in the hippocampus (Pieroza et al., 2020). These data confirmed a regional specification of NSCs including distinct molecular control, cell division modes and susceptibility to toxicants (Obner and Alvarez-Buylla, 2019). Considering that perturbations during neurogenesis could lead to long-term effects related to senility and neurodegenerative diseases (Babcock et al., 2021; de Pablo-Fernández et al., 2019; Horgusluoglu et al., 2017), BMAA could act as a neurodevelopmental toxin and promote the onset of neurological



**Fig. 6.** Effects of BMAA on activation status of ACSA2<sup>+</sup> and microglial cells in neural stem cells (NSCs) culture. Cell populations and activation status were determined by flow cytometry. A gate was first created on non-debris populations, excluding very low SSC/FSC events; cells are then gated on single cells and selected by Live/Dead staining (A). Astrocytes and B1 cells were gated as ACSA2<sup>+</sup> positive; activation status was determined through CD86 and CD206 staining (B). Quantitative analysis of the population of ACSA2<sup>+</sup> cells and the ratio of CD86<sup>+</sup> cells on CD206<sup>+</sup> cells after BMAA (100 µM) treatment as compared to untreated controls (C). Microglial cells were gated as CD11b<sup>high</sup> and CD45<sup>low/medium</sup>, activation status was determined through CD86 and CD206 staining (D). Quantitative analysis of the population of microglial cells and the ratio of CD86<sup>+</sup> cells on CD206<sup>+</sup> cells after treatment BMAA (100 µM) treatment as compared to untreated controls (E). Values are normalized and represent mean ± SEM (CTRL n = 13; BMAA 100 µM n = 9). Statistical differences from control are indicated: \*\* p < 0.001; \*\*\* p < 0.0001 using Mann-Whitney test.



**Fig. 7.** Effects of BMAA on microglial cells morphology in neural stem cells (NSCs) culture. Cell morphology was determined by immunocytochemistry; Representative microscopic photography of Iba1 staining to visualize microglia; Scale bar = 20 µm (A). Quantitative analysis of microglial cell morphology using Image-J software. Each boxplot summarizes the measurement of each microglial cells in one experimental conditions of each cell culture; area, perimeter, solidity and circularity were analyzed after BMAA (100 µM) treatment as compared to untreated controls (B). Values represent mean ± SEM (CTRL n = 44; BMAA 100 µM n = 102). Statistical differences from control are indicated: \*\*\* p < 0.001; \*\*\*\* p < 0.0001 using Mann-Whitney test.



**Fig. 8.** Effects of BMAA on proinflammatory cytokines release in neural stem cells (NSCs) culture. Levels of cytokines IL-1β, IL-6, TNFα release in the supernatant after one week of culture in the absence or presence of BMAA (100 µM) were measured by multiplex. For positive control, cells were treated during four hours at 100 ng/mL with LPS. Values represent mean ± SEM. IL-1β: CTRL n = 4; BMAA 100 µM n = 4; LPS n = 3. IL-6: CTRL n = 7; BMAA 100 µM n = 9; LPS n = 5. TNFα CTRL n = 7; BMAA 100 µM n = 9; LPS n = 8. Statistical differences from control are indicated: \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001 using Mann-Whitney test.

disorders later in life.

In addition, BMAA also acts on the migration of neuroblasts in rodents. Neuroblasts, which are neuronal precursors, migrate toward the olfactory bulb to become mature local interneurons in mice. *In vivo* this migration occurs throughout the lateral wall of the lateral ventricle, where the precursors form specific networks are organized in chains until they reach the olfactory bulb (Alvarez-Buylla, 1997). Here, we highlight for the first time that BMAA modifies neuroblast organization

by increasing the number of clusters. Neuroblasts clustering has already been observed in response to injury (Masuda et al., 2007; Zamboni et al., 2020). These grouping may correspond *in vivo* to disorganization, which can lead to disturbance of migration as we have shown in the laboratory for a pesticide, a structural analog of glutamate (Herzine et al., 2016). The mechanisms leading to these alterations involve the amino acid analogue character of these compounds, which moreover has the analogue character of a neurotransmitter in both cases. The

neurotransmitter amino-acid glutamate is of crucial importance during the migration of neuronal precursor cells from the SVZ to the olfactory bulb (Platel et al., 2010). BMAA is defined as an amino acid analog and is an agonist of metabotropic glutamate receptors (mGluR) (Aronica et al., 1993). Considering this, we hypothesized that BMAA could affect neuroblast migration by misincorporating into proteins, as a substitute of amino acid (Korn et al., 2020). In addition, BMAA can interfere with glutamate signalling and thus with SVZ functions. Indeed, di Giorgi Gerevini et al. (2004); di Giorgi-Gerevini et al. (2005) highlighted mGluR receptors expression in NSCs. Such alteration of neuroblasts organization has been reported in pathologies such as Autism Spectrum Disorders (ASD) and AD (Scopa et al., 2020; Wegiel et al., 2010). Our results confirm that BMAA could lead to abnormal neurogenesis and act as a neurodevelopmental toxin. However further *in vivo* experiments are needed to link early exposition to BMAA with late neuropathologies.

On another side, neuroinflammation can disrupt neurodevelopment processes. It has been previously described that inflammation can disturb neuroblasts migration and organization (Ekdahl et al., 2009; Tepavčević et al., 2011; Fritze et al., 2020). During development, neuroinflammation leads to a greater incidence of neurodevelopmental disorders, such as ASD, schizophrenia and epilepsy (Pantelis et al., 2021). Here, we showed that 100  $\mu$ M of BMAA drives astrocytes and microglia toward proinflammatory status in SVZ derived NSCs. Mannose receptor/CD206 expression is a hallmark of anti-inflammatory immunocompetent cells while CD86 receptor characterizes proinflammatory activation status (Smith et al., 2016). Here we report a higher ratio of CD86/CD206 of those immunocompetent cells represented by astrocytes and microglia after BMAA exposure. We hypothesized that BMAA might also affect neuroblasts migration by inducing astrocytes and microglia in proinflammatory status.

During neuroinflammatory processes the physiological activity of microglia is modified (Borst et al., 2021). In a resting state, microglia are involved in surveying their microenvironment thanks to motile processes and ramifications (Nimmerjahn et al., 2005). However after injury, microglial cells are activated and exert neurotoxic or neuroprotective effects, depending on their phenotype (Butovsky et al., 2015; Le et al., 2016). Here, we also report morphological modifications such as ameboid (or spherical) morphology, characteristics of proinflammatory activation status (Kettenmann et al., 2011; Vogel et al., 2014). However it's interesting to notice that this shape is prominent during CNS development without inducing neuroinflammation (Alliot et al., 1999). *In vitro* and *in vivo* ameboids activated microglia closely interact with NSCs residing in distinct neurogenic niches. Activated microglia have differential effects on the NSCs proliferation, migration and differentiation (Vay et al., 2018). Nevertheless, the ameboid shape of microglial cells can be associated with the release of proinflammatory cytokines (Hurley et al., 1999). As a positive control, acute exposure to LPS showed the ability of our cell culture to release proinflammatory cytokines. Our data demonstrate an increased release of the proinflammatory cytokines IL-1 $\beta$ , IL-6 and TNF $\alpha$  following chronic BMAA exposure suggesting that neuroinflammation will disturb cellular homeostasis. Among them, TNF $\alpha$  is known for enhancing the survival of neural stem cells and thereby modeling repair after inflammation (Bernardino et al., 2008). In fact, NSCs are known to proliferate in an inflammatory context to inhibit the inflammation (Meng et al., 2011; Borhani-Haghighi and Mohamadi, 2021). We have shown that BMAA promotes the proliferation of Nestin<sup>+</sup> cells in primary NSCs culture. Hence, we could speculate this proliferation of undifferentiated cells could be due to the inflammatory process via TNF $\alpha$  in order to reduce inflammation.

Several neurotoxic mechanisms induced by BMAA have been put forward. Among these, BMAA has been characterized as a non-proteinogenic amino acid that can be misincorporated into proteins (Dunlop et al., 2013; Murch et al., 2004; Glover et al., 2014). This leads to endoplasmic reticulum stress and redox imbalance (Main et al., 2016). Nevertheless, pathways of BMAA-induced inflammation remain

to be explored. However, Silva et al. (2020) report BMAA exposure resulted in elevated TLR4 and TLR3 protein, and enhanced transcription of NLRP3 and pro-IL-1 $\beta$  in cortical neurons. This augmenting effect of BMAA exposure provides the foundation for the hypothesis that BMAA induces neuroinflammation. Otherwise as an analog of glutamate, BMAA can activate mGluR which are expressed in astrocytes and microglia (Aronica et al., 1993). Interestingly, activation of mGluR5 significantly attenuates immunocompetent cells activation and reduces the LPS-induced response (Bhat et al., 2021; Byrnes et al., 2009). Considering that carbamylated forms of BMAA are analogues of glutamate, it would be interesting to study the effect of BMAA in a proinflammatory context. Further experiments are needed to determine if BMAA would increase or dampen the inflammatory response during a bacterial or viral inflammatory challenge.

## 5. Conclusions

Our study documents that BMAA-induced neuroinflammation. Here we demonstrated that BMAA has adverse effects on SVZ-derived populations and functions. We also highlighted that BMAA drives astrocytes and more drastically microglia cells into proinflammatory status. The proinflammatory effects of BMAA are one of the first steps in the BMAA toxicity cascade and result in the neuronal losses observed in the neuropathologies associated with this cyanotoxin. In fact, our study shows that BAA BMAA disrupts neuroinflammation in our model of neural stem cells culture. These data support the hypothesis of Song et al. (2016) that release of neurotoxic mediators such as cytokines could induce motor neuron loss. Nevertheless, further studies are necessary to understand precisely the etiology of sporadic neurological diseases induced by pollutants such as BMAA, by paying particular attention to the exposure. Indeed, due to climate change and environmental anthropization, cyanobacterial blooms are becoming more frequent and thus the presence of BMAA increases worldwide (Havens et al., 2003; Heisler et al., 2008; Paerl and Otten, 2013). Considering the bioaccumulation of BMAA along the trophic chain, the present study supports that BMAA could be harmful for the brain, notably through neuroinflammation.

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## CRediT authorship contribution statement

**Sarah Méresse:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Vanessa Larrigaldie:** Investigation, Writing – review & editing. **Asma Oummadi:** Resources. **Vidian de Concini:** Investigation. **Séverine Morisset-Lopez:** Investigation. **Flora Reverchon:** Methodology, Writing – review & editing. **Arnaud Menuet:** Methodology, Writing – review & editing. **Céline Montécot-Dubourg:** Writing – review & editing. **Stéphane Mortaud:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



## Data Availability

Data will be made available on request.

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## Evaluation des effets de l'inflammation combinée à une exposition chronique et combinée à des agents toxiques issus de l'environnement pendant la période périnatale : Un potentiel facteur étiologique des pathologies dégénératives

L'exposition chronique à des agents toxiques issus de l'environnement, tels que les métaux lourds, les pesticides ou les cyanotoxines, est de plus en plus rapportée comme étant un potentiel facteur sporadique des maladies dégénératives des motoneurons (MDMs). Cependant, peu de choses sont connues quant aux rôles de ces toxines dans l'étiologie des MDMs suite à une exposition précoce (ex : durant les périodes pré et post-natales du développement). Ces stades développementaux sont connus pour être des périodes critiques du développement cérébral durant lesquels des perturbations de processus fortement régulés peuvent avoir un impact négatif sur la structure fondamentale du cerveau. De telles modifications peuvent conduire au développement de vulnérabilités sélectives à la neurodégénérescence et être finalement responsables de maladies neurodégénératives à déclenchement tardif telles que les MDMs. Compte tenu du nombre croissant de preuves épidémiologiques, cliniques et expérimentales renforçant le lien entre l'exposition à des substances toxiques de l'environnement et l'apparition tardive des MDMs, il est primordial de mettre en œuvre des études expérimentales plus en adéquation avec la réalité. Par exemple, il s'agit d'étudier l'exposition précoce à des cocktails de substances toxiques pour l'environnement, à de faibles doses, pouvant induire ou prédisposer des individus aux MDMs plus tard dans la vie. Ce projet de thèse s'est focalisé sur l'étude comportementale chez la souris suite à une exposition périnatale à un mélange de différentes substances toxiques pour l'environnement à faibles doses (Glufosinate d'Ammonium (GLA),  $\beta$ -N-méthylamino-L-alanine (BMAA) et Glyphosate (GLY)), afin de vérifier si ces derniers peuvent agir en synergie et précipiter l'apparition des symptômes des MDMs plus tard dans la vie. Nous avons également vérifié si le mélange induit plus de dommages que chaque composé pris individuellement. Dans le cadre d'une hypothèse en adéquation avec la notion d'exposome, il a également été évalué l'importance et l'impact d'une sensibilisation inflammatoire asymptomatique pendant la gestation a été évalué en association à une exposition post natale aux polluants d'intérêts. Nos données révèlent qu'une exposition maternelle à au cocktail induit des perturbations des comportements moteur et anxieux chez la descendance, depuis l'adolescence ce maintenant au cours du vieillissement, ce qui n'est pas le cas lors d'une exposition aux composé pris individuellement. De plus, nous avons pu montrer que l'exposition à un seul facteur de risque (sensibilisation maternelle à de faible dose de LPS) n'était pas suffisante pour induire des modifications précoces, perdurant au cours du vieillissement, du comportement chez la descendance. Cependant, une sensibilisation inflammatoire asymptomatique associé à l'exposition ultérieure à de faibles doses de xénobiotiques, induit des effets synergiques sur le comportement des petits. Ainsi, l'ensemble de ce travail a conduit à la démonstration de l'importance de considérer la multi exposition dans la caractérisation de molécules au potentiel neurotoxique à court, moyen et long terme.

**Mots-clés :** neurodéveloppement / inflammation / polluants environnementaux/ multi-hit / pathologies neurodégénératives

## Evaluation of the effects of inflammation and combined exposure to environmental toxicants during the perinatal period: A potential etiological factor of neurodegenerative pathologies?

Chronic exposure to environmental toxic agents, such as heavy metals, pesticides or cyanotoxins, is increasingly reported to be a sporadic factor in motor neuron degenerative diseases (MNDs). However, little is known about the roles of these toxins in the etiology of MNDs following early exposure (e.g., during pre- and post-natal developmental periods). These developmental stages are known to be critical periods of brain development during which highly regulated process disturbances can negatively affect the fundamental structure of the brain. Such changes can lead to the development of selective vulnerabilities to neurodegeneration and ultimately be responsible for late-onset neurodegenerative diseases such as MNDs. Given the increasing amount of epidemiological, clinical and experimental evidence reinforcing the link between exposure to environmental toxic substances and the late onset of MNDs, it is essential to implement experimental studies closer to actual exposure conditions. For example, early exposure to cocktails of substances that are toxic to the environment, at low doses, may induce or predispose individuals to MNDs later in life. This thesis project focused on a behavioural study in mice following a perinatal exposure to a mixture of various environmental toxic substances at low doses (Glufosinate ammonium (GLA),  $\beta$ -N-methylamino-L-alanine (BMAA) and Glyphosate (GLY)), to see if they can act synergistically and precipitate the appearance of symptoms of MNDs later in life. We also checked whether the mixture induces more damage than each compound individually. Based on this hypothesis and in line with "exposome" notion, it was also assessed the importance of an asymptomatic inflammatory sensitization during pregnancy in combination with post-natal exposure to pollutants of interest. Our data revealed that a maternal exposure to this cocktail induces disturbances in motor and anxiety behaviours in offspring, from adolescence to aging. Interestingly, these impairments have not been observed when individuals were exposed to each compound separately. In addition, we were able to show that an exposure to a single risk factor such as prenatal immune sensitization to low dose of LPS is not sufficient to induce early and long lasting behavioural changes in offspring's. However, we demonstrated that this asymptomatic inflammatory sensitization associated to subsequent low doses xenobiotic exposure, act in synergy to affect behaviour in offspring's. Thus, these complementary studies have led to the demonstration of the importance of considering multi-exposure in the characterization of molecules with a neurotoxic potential in the short, medium and long term.

**Key words:** neurodevelopment / inflammation / environmental pollutants / multiple-hit / neurodegenerative diseases