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# Inflammation and immune-mediated neurobehavioral alterations: a critical role for microglia

Chloé Lacabanne

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THÈSE EN COTUTELLE PRÉSENTÉE  
POUR OBTENIR LE GRADE DE  
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ÉCOLE DOCTORALE UBX  
INTEGRATED PROGRAM IN NEUROSCIENCE

Par Chloé Lacabanne

**INFLAMMATION AND IMMUNE-MEDIATED  
NEUROBEHAVIOURAL ALTERATIONS:  
A CRITICAL ROLE FOR MICROGLIA**

Sous la direction du Dr. Sophie Layé  
et du Dr. Giamal Luheshi

Pr. Pierre Gressens, Full Professor, INSERM, Paris, France - Nominated external examiner  
Pr. Lalit Srivastava, Full professor, McGill University, Montreal, Canada - Internal examiner  
Dr. Steve Lacroix, Research Director, Université de Laval, Québec, Canada - External examiner  
Dr. Thierry Alquier, Research Director, Université de Montréal, Québec, Canada - External examiner  
Dr. David Gosselin, Research Director, Université de Laval, Québec, Canada - External examiner  
Pr. Josephine Nalbantoglu, Full professor, McGill University, Montreal, Canada - Invité

## Résumé

Les cellules microgliales sont les cellules de l'immunité innée résidentes du cerveau. Elles jouent un rôle important d'une part dans la réponse inflammatoire cérébrale et d'autre part, dans la formation et le remodelage des réseaux neuronaux lors du développement cérébral. De par le répertoire de récepteurs qu'elles possèdent et leur capacité à répondre de façon rapide et dynamique aux modifications de leur environnement, les cellules microgliales sont particulièrement sensibles aux stimuli environnementaux. Dans certaines situations, des réponses inadaptées aux stimuli externes entraînent des modifications de leurs activités physiologiques et une altération de la mise en place des réseaux neuronaux et des altérations psychopathologiques. Dans ce travail de thèse nous avons étudié le rôle des cellules microgliales dans les effets neurobiologiques et comportementaux d'un stimulus inflammatoire administré à l'âge adulte ou au cours du développement cérébral.

Des travaux précédemment conduit au sein des laboratoires d'accueil ont mis en évidence que l'administration périphérique de lipopolysaccharide (LPS), une endotoxine bactérienne, provoque l'apparition d'altérations comportementales de type anxieux et dépressifs. Cependant, le rôle des cellules microgliales dans les altérations comportementales provoquées par une inflammation n'étant pas connu, il a fait l'objet de la première étude présentée dans mon travail de thèse (Chapitre 2). Afin d'éliminer les cellules microgliales du cerveau, les souris ont reçu par voie d'administration intra-hippocampique (région CA3), des liposomes contenant du clodronate qui, après phagocytose, provoque leur apoptose. Chez les souris contrôle (sans clodronate), l'administration de LPS induit dans l'hippocampe la synthèse de cytokines pro-inflammatoires [interleukine (IL)-1 $\beta$  et facteur de nécrose tumorale (TNF)- $\alpha$ ] et anti-inflammatoires (IL-10)]. Le LPS induit également la synthèse de

l'indoleamine 2,3-dioxygénase (IDO), une enzyme impliquée dans le métabolisme du tryptophane (le précurseur de la sérotonine) en kynurénine, dont les métabolites ont des effets toxiques sur les fonctions cérébrales. L'expression de IDO dans le cerveau est utilisée comme un biomarqueur de la dépression induite par l'inflammation chez les rongeurs. La production induite du  $\text{TNF}\alpha$ , de l'IL-10 et de l'IDO, mais pas de l'IL-1 $\beta$ , sont significativement diminués dans l'hippocampe de souris traitées par le clodronate, suggérant que la déplétion des microglies joue un rôle dans la réponse neuroinflammatoire au LPS. Les effets comportementaux du LPS, mesurés par le test de nage forcé, montrent que le LPS provoque des comportements de type dépressifs, qui sont atténués par le clodronate. Dans leur ensemble, ces résultats indiquent que les effets comportementaux et neuroinflammatoires du LPS impliquent les cellules microgliales.

Sur la base de ces résultats et compte tenu du rôle récemment décrit de la microglie dans le développement des réseaux neuronaux, nous avons étudié l'effet à long terme d'une activation immunitaire maternelle sur la population de microglie et le comportement de la progéniture. Pour cela, nous avons utilisé un modèle d'activation immunitaire maternelle en utilisant le même LPS que celui utilisé au chapitre 2, administré à des souris à un stade relativement précoce de la gestation, lorsque la microglie commence à coloniser le cerveau fœtal. Nous avons recherché si les cellules microgliales sont impliquées dans les effets du LPS, administré au cours de la gestation, sur les altérations comportementales qui se développent à long terme chez la descendance. Les cellules microgliales colonisent le cerveau en développement dès les premiers stades de vie embryonnaire et l'effet d'un stimulus inflammatoire sur leurs activités n'est pas connu. Nous avons développé un modèle murin d'inflammation maternelle, étudié l'impact de cette inflammation précoce sur les cellules microgliales de la progéniture durant le développement embryonnaire, la période périnatale et à l'âge adulte, ainsi que les conséquences comportementales de

cet épisode inflammatoire précoce sur le comportement à long terme de la descendance (Chapitres 3 & 4). Plus spécifiquement, nous avons étudié les effets de l'administration de LPS à des souris gestantes au jour de vie embryonnaire (JG) 9.5 sur la trajectoire développementale pré et post-natale des cellules microgliales et du comportement post-natal de la progéniture (Chapitre 3). L'administration de LPS à JG 9.5 n'affecte pas le pourcentage total de cellules microgliales mais entraîne une réduction du pourcentage de microglies « matures » aux JG14.5 et 18.5. Des mesures telles que la géotaxie négative et le réflexe de retournement ont permis de montrer que le LPS prénatal n'affecte pas le développement sensori-moteur des souriceaux. Le LPS prénatal entraîne des déficits de vocalisation durant la période post-natale, qui sont des indicateurs d'altération de comportement émotionnel. Des déficits comportementaux persistent à l'âge adulte, avec un dimorphisme sexuel prononcé. En effet, au jour postnatal (JPN)65, les souris mâles dont la mère a reçu une administration de LPS durant la gestation présentent une augmentation des comportements répétitifs ainsi qu'une diminution des comportements sociaux tandis que les femelles des mêmes fratries ne présentent pas d'altérations comportementales. A l'inverse, la mémoire sociale est altérée par le LPS prénatal uniquement chez les femelles de la descendance.

Dans un troisième temps, nous avons recherché les mécanismes moléculaires impliqués dans les effets comportementaux du LPS administré à JG9.5, afin de déterminer si les cytokines inflammatoires étaient l'intermédiaire moléculaire entre les modifications phénotypiques microgliales au cours du développement et les altérations comportementales qui se développent à l'âge adulte, et ce chez les mâles et les femelles (Chapitre 4). Pour cela, nous nous sommes focalisés sur le rôle de l'IL-1 $\beta$ , qui est la cytokine inflammatoire effectrice principale de l'activité microgliale induite par le LPS. L'expression de l'IL-1 $\beta$  et des cytokines associées (IL-6, TNF $\alpha$  et IL-10) augmente significativement dans le plasma maternel, le placenta et le cerveau

foetal, 2 et 4 heures après l'administration de LPS. Ces changements sont accompagnés d'une diminution du pourcentage de microglies matures à JG18.5 et de la population microgliale totale JPN9. À l'âge adulte (JPN65), nous avons observé une augmentation de la densité et des changements morphologiques de la microglie dans des structures cérébrales impliquées dans les comportements mesurés. Enfin, les souris adultes développent des altérations des comportements de type sociaux, de la mémoire sociale et des comportements répétitifs. Les changements du nombre de microglies étaient significativement corrélés aux troubles comportementaux, et ce de façon spécifique en fonction du sexe des souris. La co-administration de l'antagoniste du récepteur de l'IL-1 (ra) avec le LPS chez les femelles gestantes au JG9.5 a significativement réduit les effets inflammatoires et comportementaux du LPS sur la descendance.

En conclusion, ce travail de thèse montre l'implication de la microglie dans les effets comportementaux d'une inflammation périphérique à court terme provoquée chez des souris adulte ou à long terme chez la descendance. Ainsi, d'une part la déplétion des cellules microgliales de souris adulte prévient les comportements dépressifs provoqués par le LPS. D'autre part, nos résultats mettent en évidence pour la première fois que la perturbation de la trajectoire développementale des cellules microgliales provoquée par une inflammation prénatale précoce et les conséquences comportementales qui se développent à l'âge adulte présentent un dimorphisme sexuel et implique l'IL-1 $\beta$ .

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## **Abstract**

Recent research on microglia has uncovered a multitude of activities that extends the role of these cells well beyond their traditional function as immune sentinels. The most prominent of these newly described activities is an intricate role in neuronal network remodeling notably upon environmental challenge or during brain development, the disruption of which can result in long lasting consequences relevant to several psychopathologies. We sought, in the current thesis, to identify some of the mechanisms involved.

Our initial approach was to target the immune function of microglia, based on our previous findings linking systemic immunogenic challenge with lipopolysaccharide (LPS) in mice with the development of despair-like behaviour/depression. Here, we sought to identify immune mediators activated in microglia following a single systemic challenge with LPS (Chapter 2). These studies were conducted in adult mice in which phagocytic microglia were depleted using a single injection of liposomal clodronate in the CA3 region of the hippocampus. LPS challenge significantly upregulated the expression of both pro-inflammatory [interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF) $\alpha$ ] and anti-inflammatory (IL-10) cytokines compared to saline treated animals. In addition, LPS highly increased the expression of indoleamine 2,3-dioxygenase (IDO), an important rate limiting enzyme for metabolizing tryptophan in the brain and an established indicator of the activation of this depression mediating pathway. Clodronate-mediated depletion attenuated all of these effects apart from IL-1 $\beta$  expression which was further exacerbated. Behavioural assessment of the mice demonstrated a significant LPS-induced increase of immobility in the forced swim test (FST), which was prevented by clodronate. This experimental approach provided a snapshot of the role of inflammation in the development of brain dysfunction mediated by microglia. In subsequent studies (chapter 3 & 4), and in order to perform a more comprehensive, longer-term investigation of microglia activity in neurodevelopment, we utilized a prenatal infection model using LPS to activate maternal immunity at a relatively early [Gestational Day (GD)9.5] time point when microglia colonize the fetal brain to assess the impact on microglial population during development and the subsequent behaviour of the progeny

(Chapter 3). The results demonstrated LPS reduced the percentage of mature microglial population at GD14.5 and GD18.5 representing mid to late gestation. In addition, prenatal LPS had a significant effect on the offspring's neonatal as well as adult behaviour, with a clear divergence along sex lines in adulthood.

In the final study (Chapter 4), we sought to investigate the mechanisms underlying the changes we noted in microglial development and the sexually dimorphic behavioural deficits. For this, we focused on the role played by pro-inflammatory cytokines, particularly IL-1 $\beta$  which represents the main effector of microglial activation following infection or injury. Detailed analysis of the expression of IL-1 $\beta$  and other related cytokines (IL-6, TNF $\alpha$  and IL-10) revealed an increased expression of these mediators in maternal plasma, placenta and fetal brain, 2 and 4 hours after the prenatal LPS treatment. These changes were accompanied with a decreased percentage of mature microglia in the brain of embryos at GD18.5 and of total microglia population at post-natal day (PND)9. In the adult offspring (PND65), we detected an increased density and altered microglial morphology in specific higher-order structures implicated in complex behaviours, as well as altered social preference and memory and increased repetitive actions. Interestingly, changes in the number of microglia correlated significantly with the LPS-induced behavioural impairments, most of which were sexually dimorphic with a bias towards males. Strikingly, co-administration of the IL-1 receptor antagonist (ra) in mice at GD9.5 either prevented or significantly attenuated the effects induced by LPS at all the developmental time points tested.

In conclusion, the work presented in this thesis shows the role of microglia in mediating short- and long-term LPS-induced behavioural deficits. Notably, our results show that depletion of phagocytic microglia can attenuate LPS-induced despair-like behaviours while LPS-induced inflammation during embryonic development leads to the exhibition of sexually dimorphic traits and disrupted microglial development for which IL-1 $\beta$  appears to be the primary mediator.

**Keywords:** Microglia – LPS – Inflammation – Neurodevelopment – Neurobehavioural alterations – IL-1 $\beta$

## Résumé

Les microglies, les cellules de l'immunité innée, résidentes du cerveau, sont impliquées dans la réponse inflammatoire cérébrale et le modelage des réseaux neuronaux au cours du développement. La perturbation de leurs activités par des stimuli environnementaux pouvant conduire à des altérations psychopathologiques, dans cette thèse, nous avons étudié le rôle des microglies dans les effets neurobiologiques et comportementaux d'un stimulus inflammatoire.

Les travaux précédents ont révélé que l'administration de lipopolysaccharide (LPS), une endotoxine bactérienne, provoque des comportements de type dépressifs. Le rôle des microglies dans ces altérations a fait l'objet de la première étude de cette thèse (Chapitre 2). Afin de dépléter les microglies du cerveau, des souris adultes ont reçu par voie d'administration intra-hippocampique des liposomes contenant du clodronate, provoquant ainsi l'apoptose des microglies phagocytaires. L'administration de LPS active dans l'hippocampe la synthèse de cytokines pro-inflammatoires [interleukin (IL)-1 $\beta$  et facteur de nécrose tumorale (TNF) $\alpha$ ] et anti-inflammatoires (IL-10), et de l'indoleamine 2,3-dioxygénase, une enzyme impliquée dans le métabolisme du tryptophane aux activités pro-dépressives. La déplétion des microglies phagocytaires atténue les effets du LPS, à l'exception de l'IL-1 $\beta$ , dont l'expression est exacerbée. De plus, l'administration de clodronate prévient les effets du LPS sur les comportements de type dépressif. Dans leur ensemble, ces résultats ont révélé que les microglies phagocytaires sont impliquées dans les effets inflammatoires et comportementaux de type dépressif induits par le LPS.

Nous avons ensuite étudié le rôle des microglies dans les effets comportementaux d'une inflammation maternelle précoce provoquée lors de la colonisation du cerveau fœtal par les microglies (Chapitres 3 & 4). Ainsi, nous avons administré au jour gestationnel (JG)9.5 du LPS à des souris gestantes et évalué la trajectoire développementale pré- et post-natale des microglies et du comportement de la progéniture (Chapitre 3). L'administration de LPS à JG9.5 provoque une réduction du pourcentage représenté par les microglies matures aux

JG14.5 et 18.5 et des déficits comportementaux persistants à l'âge adulte avec un dimorphisme sexuel prononcé.

Nous avons alors recherché à identifier les mécanismes moléculaires impliqués dans les effets du LPS administré à JG9.5, en étudiant la piste de l'action des cytokines inflammatoires (Chapitre 4). Pour cela, nous nous sommes focalisés sur l'IL-1 $\beta$ , la cytokine inflammatoire effectrice principale de l'activité microgliale. L'expression de l'IL-1 $\beta$  et des cytokines associées (IL-6, TNF $\alpha$  et IL-10) augmente dans le plasma maternel, le placenta et le cerveau fœtal, 2 et 4 heures après l'administration de LPS. Ces changements sont accompagnés d'un phénotype microglial immature à JG18.5 et d'une réduction de la population microgliale totale au jour postnatal (JPN)9. À l'âge adulte (JPN65), nous avons observé une modification morphologique de la microglie dans plusieurs structures cérébrales. Enfin, les souris adultes, prénatalement traitées au LPS, développent des altérations des comportements de type sociaux et des comportements répétitifs. Les altérations du nombre de microglies induites par le LPS sont corrélées aux troubles comportementaux, et ce, de façon spécifique en fonction du sexe des souris. Enfin, la co-administration de l'antagoniste du récepteur de l'IL-1 et de LPS chez les femelles gestantes au JG9.5 réduit, voire prévient les effets inflammatoires et comportementaux du LPS.

En conclusion, ce travail de thèse montre l'implication des microglies dans les effets comportementaux d'une inflammation périphérique à court terme chez des souris adultes, ou à long terme chez la descendance de souris ayant subi une activation immunitaire durant la gestation. D'une part, la déplétion des microglies phagocytaires prévient les comportements dépressifs induits par le LPS. D'autre part, nos résultats mettent en évidence pour la première fois que la perturbation de la trajectoire développementale microgliale induite par une inflammation prénatale, ainsi que les conséquences comportementales qui se développent à l'âge adulte, impliquent IL-1 $\beta$ .

**Mots clés :** Microglie – LPS – Inflammation – Neurodéveloppement – Altérations neurocomportementales – IL-1 $\beta$

## Contribution of authors

### **Chapter II-Research Manuscript:**

Design of the study: C. Lacabanne, S. Layé and G. N. Luheshi

Execution and analysis of the study: C. Lacabanne, A. Benmamar-Badel, Q. Leyrolle

Writing of the manuscript: C. Lacabanne, S. Layé and G. N. Luheshi

Supervision: S. Layé and G. N. Luheshi

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### **Chapter III-Research Manuscript:**

Design of the study: C. Lacabanne, S. Layé and G. N. Luheshi

Execution and analysis of the study: C. Lacabanne, M. Bordeleau

Writing of the manuscript: C. Lacabanne, S. Layé and G. N. Luheshi

Supervision: S. Layé and G. N. Luheshi

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### **Chapter IV-Research Manuscript:**

Design of the study: C. Lacabanne, S. Layé and G. N. Luheshi

Execution and analysis of the study: C. Lacabanne, L. Fernandez de Cossío

Collaborators providing unique reagents: S. Allan

Writing of the manuscript: C. Lacabanne, S. Layé and G. N. Luheshi

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## Introduction

Microglia, the resident innate immune cells in the brain, constitute the first line of defence against brain injury and infection. Their vast repertoire of pattern recognition receptors (PRR) that include such family members as Toll-like receptors and phagocytic receptors, collectively function to sense and eliminate microbes invading the central nervous system (CNS) parenchyma. In addition, activation of these cells which continuously survey their immediate environment for intricate changes, elicits a broad range of inflammatory mediators that are involved in the recruitment and subsequent activation of peripheral immune cells infiltrating the infected CNS [see (Mariani and Kielian 2009) for review]. The majority of these mediators belong to the cytokine family and are defined as either pro- or anti-inflammatory depending on their activity [see (Cavaillon 2001) for review]. Although the binary concept of microglial M1/M2 classification has been recently debated (Ransohoff 2016), microglia activation state can also be defined as pro-inflammatory and neurodegenerative (M1) or anti-inflammatory and neuroprotective (M2) and both play a role in the resolution of an immune challenge (Chhor et al. 2013; Orihuela, McPherson, and Harry 2016). Upon appropriate stimulation, classically activated, M1 microglia produce pro-inflammatory cytokines [*e.g.* interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF) $\alpha$ ], in order to kill the offending foreign pathogen and polarize T-cells to mount an adaptive immune response (Gordon and Taylor 2005; Villalta et al. 2009). M2 macrophages facilitate resolution of inflammation through anti-inflammatory factors (*e.g.* IL-10), to deactivate pro-inflammatory cell phenotypes and re-establish homeostasis (Gordon 2003; Gordon and Martinez 2010; Ortega-Gómez, Perretti, and Soehnlein 2013). This type of response mounted by microglia is not limited to local injury or brain infection but can just as efficiently be triggered following systemic inflammation. Experimental approaches using immunogenic stimuli such as bacterial lipopolysaccharide (LPS) in rodents (Hoogland et al. 2015, 2018) and humans (Sandiego et al. 2015) clearly demonstrate a robust response mounted by microglia and mediated by brain borne cytokines, that includes the transition from M1 to M2 phenotypes. As well as activating the microglia, systemic LPS, is used to trigger an array of

sickness type responses that include fever, appetite suppression, loss of interest in immediate environment and malaise which in some cases can develop into a more chronic state of despair and depression (Bay-Richter et al. 2011; Dantzer et al. 2008; Frenois et al. 2007; O'Connor et al. 2009; Sekio and Seki 2015). We and others have previously investigated the role of microglia in mounting and resolving these responses and whilst a direct link has not been clearly described, numerous evidence, including some from our laboratory (Aguilar-Valles et al. 2014; Delpech et al. 2016; Henry et al. 2008; Yeh et al. 2018) do suggest that microglia are key to the triggering and progression of these types of sickness-like behaviours including depression.

In the first results chapter (**Chapter 2**) of the present thesis, we addressed the *hypothesis* that *microglial polarization triggered by LPS will alter the ensuing inflammatory response and subsequently the behavioural outcomes in adult mice*. For this study, we specifically depleted M2 microglia in the hippocampi *via* an intra-hippocampal administration of liposomes containing dichloromethylene-bisphosphonate (clodronate), inducing apoptosis of phagocytic microglia. The animals were then challenged with LPS to determine the level of influence that M2 microglia have on the development of both the inflammatory response and the despair-like behaviours measured in our previous study (Aguilar-Valles et al. 2014). The results from this work show that depletion of M2 microglia decreases LPS-induced expression levels of IL-10 and TNF $\alpha$  while it potentiates LPS-induced expression levels of the pro-inflammatory cytokine IL-1 $\beta$ . In addition, increase of indoleamine 2,3-dioxygenase (IDO), the metabolic rate limiting enzyme of the tryptophan pathway and despair-like behaviour induced by LPS were respectively completely prevented and attenuated by the intracerebral administration of clodronate liposomes. Interestingly, LPS-induced behavioural alterations, as measured by increased immobility time in forced swim test and IDO expression levels were highly correlated. Altogether, these data suggest that phagocytic microglia may be important effectors underlying the inflammatory changes associated with depressive-like behaviours.

The studies performed in Chapter 2 were based on established activities of microglia reflecting their “short-term” role in protecting the adult brain and in modulating sickness behaviours triggered by a systemic inflammation. The discovery that microglia have much

broader function, including modulating early neurodevelopmental processes, suggested a potential vulnerability, that maybe linked to inflammation induces neurodevelopmental defects leading to psychiatric disorders. The *specific hypothesis* here is that *affecting microglia population in early neurodevelopment, for example following inflammation, will lead to the formation of aberrant networks leading to disrupted brain development and subsequent psychopathology in the afflicted individual*. One of the roles that microglia play in the developing CNS is known as pruning and is mediated *via* the fractalkine pathway (Paolicelli et al. 2011; Schafer et al. 2012). A study using transgenic animals in which the fractalkine receptor (CX3CR1) expression is deleted exhibited deficient synaptic pruning associated with weak synaptic transmission, decreased functional brain connectivity, and behavioural alterations previously associated with autism spectrum disorders (ASD) and other neurodevelopmental and neuropsychiatric disorders (Zhan et al. 2014). Whilst this and other studies (Bilimoria and Stevens 2015; Tremblay, Lowery, and Majewska 2010; Wu et al. 2015) confirmed the importance of microglia in brain development and the critical role played by an intact fractalkine pathway, the significance of this from a clinical prospective only became prominent with the development and use of animal models to test the impact of environmental risk factors such as infection on the developing fetal brain.

The development of animal experimentation in this area was based largely on epidemiological studies showing a significantly increased incidence of neurodevelopmental disorders such as ASD (Atladóttir et al. 2010) and schizophrenia (SCZ) (Brown et al. 2004) in offspring of mothers exposed to the influenza virus during gestation. The initial research into this area, using a prenatal infection approach with different immunogenic stimuli including LPS and the viral mimic Poly(I:C), focussed on the inflammatory response triggered in the maternal plasma, fetal compartment and brain (Ashdown et al. 2006; Cai et al. 2000; Gayle 2004; Gilmore, Jarskog, and Vadlamudi 2005; Meyer 2006; Meyer et al. 2008; Urakubo et al. 2001). Using this rationale evidence was submitted, including some from our own laboratory (Ashdown et al. 2006; Fortier et al. 2004; Labrousse et al. 2018), showing a link between inflammatory events *in utero* and increased incidence of abnormal behaviours in the offspring. While presenting some advances in our understanding of potential events that may be responsible for disrupting fetal brain development and

subsequent pathology in the offspring, these results were mostly correlative. It was not until the role of microglia in neurodevelopment was described that a potential mechanism to explain the underlying causes of disrupted early brain development was suggested. Our focus, as with many others, changed towards studying microglial function using a developmental timeline and LPS as the immunogen for triggering the prenatal infection. In an earlier study (Fernández de Cossío et al. 2017), we confirmed that treatment of mothers with LPS in mid gestation [Gestational day (GD)15], disrupted the fractalkine system, increased hippocampal spine density and led to behavioural changes relevant to ASD.

In this thesis (**Chapter 3**) we were however interested in a more global approach designed to investigate the impact of an early maternal infection on microglial population during development (*in utero* and during postnatal period) and the subsequent behaviour of the offspring. For this, LPS was administered at a relatively early time point (GD9.5) identified as when microglia colonize the fetal brain (Ginhoux et al. 2010). The results from these studies show that prenatal LPS does not affect the percentage of microglia in the embryonic brain but results in a microglial population with an immature phenotype. In addition, while maternal immune activation (MIA) does not affect reflex development, neuromotor ability and olfactory discrimination, it alters social/communicative behaviour in neonatal offspring. In adulthood, prenatal LPS affects the offspring' repetitive behaviour, social preference, social memory and amphetamine-induced locomotor activity. A notable outcome of these studies is that we observed a clear division in behavioural outcomes along sex lines of the offspring. We have observed this phenomenon previously and a number of studies have shown this to apply to microglial development and function (Schwarz, Sholar, and Bilbo 2012; Vanryzin, Pickett, and Mccarthy 2018; Weinhard et al. 2018). Interestingly, sexual dimorphism has long been recognized as a feature of a number of neurodevelopmental disorders including ASD and SCZ [see (Hanamsagar and Bilbo 2016) for review] and our data combined with those of others suggest that this may be microglia driven.

We first identified that inflammatory events triggered by immunogenic stimuli contribute to molecular, cellular and to subsequent behavioural alterations (Chapter 2) and that prenatal

infection disrupts microglial population during fetal development, leading to abnormal behaviours in the offspring of infected mothers (Chapter 3).

In the final study (**Chapter 4**), we investigated the mechanisms underlying the changes we noted in microglial population during development and the sexually dimorphic behavioural deficits following prenatal administration of LPS and assessed the contribution of microglial alterations to the behavioural defects. Based on our observations that these responses appear to be influenced by the sex of the progeny, all of our studies were performed in males and females. In previous studies, we demonstrated that prenatal exposure of pregnant mothers to a single dose of LPS triggers a robust inflammatory response that is exemplified by the production of a range of pro and anti-inflammatory cytokines (Ashdown et al. 2006). Here, we focussed on one of the most prominent of these mediators, namely IL-1 $\beta$ , considered to be central to mounting the full cytokine response. In addition, we considered the demonstrated role of this cytokine as a primary regulator of microglial activity. Thus, in these studies we addressed the *hypothesis* that *the role of microglia in embryonic neurodevelopment is disrupted by a prenatal challenge with bacterial LPS through an IL-1 $\beta$  mediated process and results in behavioural alterations in a sexually dimorphic manner*. Our approach for these studies was first, to assess the effect of prenatal LPS treatment on the inflammatory response measuring levels of expression of IL-1 $\beta$  as well as its downstream mediator IL-6 in addition to TNF $\alpha$  and the anti-inflammatory cytokine IL-10, in different compartments of interest (maternal plasma, placenta and fetal brain). Results from these experiments demonstrated that LPS administration induces the expression of both pro- and anti-inflammatory cytokines in the maternal plasma, while only IL-6 and respectively TNF $\alpha$  and IL-10 were induced in the placenta and fetus. Since our central strategy in performing these studies was to assess the role of IL-1 $\beta$ , we adopted a neutralization approach using a recombinant version of the naturally occurring IL-1 receptor antagonist (ra), a competitive antagonist at the level of the IL-1 type I receptor and a very efficient method to inhibit IL-1 actions *in vivo* as was previously demonstrated by us (Cartmell et al. 1998, 2000; Cartmell, Luheshi, and Rothwell 1999; Laye et al. 1995; Luheshi et al. 1997). As expected, co-administration of this inhibitor with LPS significantly attenuated or completely prevented the LPS-induced expression of pro-inflammatory mediators (IL-1 $\beta$ , IL-6 and TNF $\alpha$ ) and

potentiated levels of the anti-inflammatory cytokine IL-10 in all compartments. The effects of IL-1ra treatment were also investigated in the changes observed on microglial population outlined in the previous chapter (Chapter 3) where IL-1ra co-administration prevented the LPS-induced orientation of microglia towards the immature phenotype at GD18.5. These results are the first to demonstrate a direct role for IL-1 $\beta$  in regulating embryonic microglial changes. Despite the significance of these findings, perhaps the most interesting result (in relative terms) emanating from these studies is that the influence of a single prenatal dose of IL-1ra appeared to have significant extended effects that were still apparent in the offspring at early postnatal period and up to adulthood. We assessed the effects of prenatal treatment on microglial population at postnatal day (PND)9 and while LPS decreases the percentage represented by microglia cells among live cells, this was prevented by IL-1ra co-administration. Similarly, at adulthood, increased density and morphological microglial changes induced by LPS were prevented by IL-1ra co-administration. Finally, all behavioural alterations measured in LPS-treated adult offspring (decreased social interaction and social memory and increased repetitive behaviour) were prevented by IL-1ra. These studies demonstrated a remarkable correlation between reduction in microglial density in several higher order cognitive-emotional structures that are implicated in complex behaviours and behavioural outcomes in the adult mice of LPS treated mothers, suggesting a strong functional association between microglial and behavioural alterations.

In sum, the work presented in the current thesis provides strong evidence that changes in microglial population are related to triggering and propagation of aberrant behaviours associated with mental disorders in humans. This influence can occur in two possible ways: first, through acute, short-term alterations in microglia postnatally, through the modulation of sickness type responses that have the potential of developing into more serious despair-like behaviour; or second through a more sustained change in microglial population *in utero* affecting the development of the fetal brain. As presented in this thesis this scenario had very serious long-term consequences with behavioural effects apparent even at adulthood. We have identified the inflammatory mechanisms underlying these longer-term changes and confirmed that a clear separation exists in the molecular cellular and behavioural alterations

along sex lines in agreement with the sexually dimorphic traits reported in numerous mental health disorders in humans.

## **Chapter I:**

### **Comprehensive review of the literature**

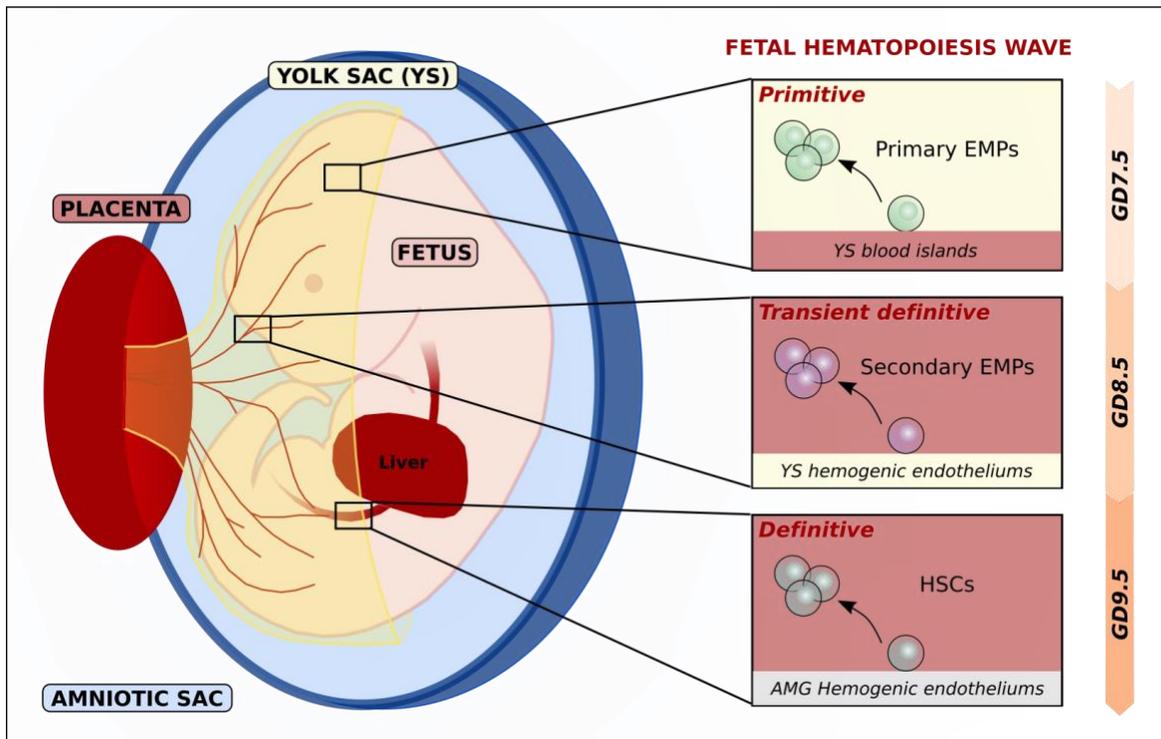
## I.1. The unique embryonic origin of microglia

### I.1.1. Ontogeny of microglia

Although Rio del Hortega already suspected a mesodermal origin of what he first introduced as “microglia” in 1932 [see (Sierra et al. 2016) for review], there was much more support for a neuro-ectodermal origin. Since then, defining the origin of microglia has been the subject of much debate for several decades. In the past few years, several lineage-tracing experiments (Ginhoux et al. 2010; Gomez Perdiguero et al. 2015; Hashimoto et al. 2013; Hoeffel et al. 2015; C. Schulz et al. 2012; Sheng, Ruedl, and Karjalainen 2015; Yona et al. 2013), together with parabiosis experiments (Ajami et al. 2007; Hashimoto et al. 2013) and neonatal bone marrow transplantations (Ginhoux et al. 2010; Hashimoto et al. 2013), systematically delineated the source of microglia and confirmed their yolk sac (YS) ontogeny (Hashimoto et al. 2013).

In rodents, there are at least three waves of hematopoiesis whose timing and tissues involved partially overlap, highlighting the challenging aspect of trying to delineate microglial ontogeny. The first wave occurs in the extra-embryonic YS blood islands at gestational day (GD)7.0 and generates “primitive” progenitor cells at approximately GD7.5. These progenitors differentiate notably into YS macrophages without passing through a monocytic stage, hence the name “primitive” hematopoiesis (Ginhoux and Guilliams 2016; Gomez Perdiguero et al. 2015). These YS primitive progenitors [which do not express the hematopoietic cells marker CD45 but express the hematopoietic stem cells marker ckit (CD45<sup>-</sup> ckit<sup>+</sup>)] are by nature primary erythromyeloid precursors (EMPs) and their descendants, the YS macrophages (which are CD45<sup>-</sup> ckit<sup>low</sup>), colonize the entire embryo including the brain to generate microglia (Ginhoux and Guilliams 2016; Hoeffel et al. 2015). Primary EMPs express macrophage colony-stimulating factor 1 receptor (CSF1R) and require signaling through this receptor for their survival and differentiation. Moreover, they are independent of the transcriptional activator MYB (Ginhoux et al. 2010; Hoeffel et al. 2015). At GD8.25, the second wave of hematopoiesis, called “transient definitive”, is initiated in the hemogenic endothelium of the YS, which generates “late” or secondary EMPs (which are CD45<sup>low</sup> ckit<sup>low</sup>). These secondary YS progenitors are also considered as

precursors of YS macrophages (Gomez Perdiguero et al. 2015; Perdiguero and Geissmann 2016). Contrary to the primary EMPs, secondary EMPs are MYB dependent; their expression of CSF1R is contested (Hoeffel et al. 2015). Overlapping with the second wave of hematopoiesis, the third “definitive” wave starts within the embryo at GD9.5, generating immature hematopoietic stem cells (HSCs) from the hemogenic endothelium in the aorto-gonado-mesophrenos region (Cumano and Godin 2007). Ultimately, these precursors colonize the fetal liver where they establish definitive hematopoiesis. Blood brain barrier (BBB) formation coincides with the timing of broad fetal liver monocyte infiltration (Daneman et al. 2010). Although direct evidence is lacking, BBB has been speculated to be the main mechanism for blocking fetal liver monocyte accessibility to the embryonic brain (Ginhoux et al. 2013; Hoeffel et al. 2015). Altogether, the current view is that microglia are generated from YS EMPs giving rise to YS macrophages, which are the precursors that colonize the embryonic brain (Gomez Perdiguero et al. 2015; Kierdorf et al. 2013), reside in the brain throughout life, and maintain their numbers through a process of self-renewal (Hashimoto et al. 2013). However, the precise nature and time of emergence of these EMPs (primary or secondary EMPs) are still under debate (Hashimoto et al. 2013; Kierdorf et al. 2015; Perdiguero and Geissmann 2016) ([Figure I.1](#)).

**Figure I.1. Embryonic hematopoiesis.**

**Figure I.1.** Three main successive waves of hematopoiesis occur during development. The first (“primitive”) arises from the yolk sac (YS) blood islands and gives rise to primary erythromyeloid precursors (EMPs) at GD7.5. The second waves (“transient definitive”) arises from the YS hemogenic endotheliums and gives rise to secondary EMPs at GD8.5. Finally, the third wave (“definitive”) occurs in the hemogenic endothelium in the aorto-gonado-mesophrenos (AMG) region and gives rise to hematopoietic stem cells (HSCs) at GD9.5. Both primary and secondary EMPs but not HSCs are microglial progenitors. Adapted from (Ginhoux and Guilliams 2016).

### ***1.1.2. Development of microglia***

#### ***- Microglial precursors migration -***

Originating in the YS, microglial progenitors (primary and secondary EMPs) differentiate into YS macrophages before migrating to the embryonic tissues, including to the brain. Experiments using CX3 C-chemokine receptor 1 (CX3CR1)-GFP mice [which express green fluorescent protein (GFP) under the control of the endogenous CX3CR1 locus] detected

microglial precursors in the cephalic mesenchyme as early as GD9.0 and later, at GD9.5, within the neuroepithelium (Ginhoux et al. 2010; Kierdorf et al. 2013). Although the precise mechanisms by which these precursors colonize the brain parenchyma remain to be clarified indirect observations suggest that normal blood circulation is essential. Indeed, interestingly, the appearance of YS precursors in the embryo coincides with *de novo* formation and remodeling of blood vessels in the mouse embryo that occurs between GD8.0 and GD10.0 (Walls et al. 2008). In a recent study, intra-vital imaging of trafficking routes of YS macrophage progenitors during embryogenesis allowed recording of YS EMPs entering the YS vasculature and travelling toward the embryo at GD9.0 (Stremmel et al. 2018). Moreover, sodium-calcium exchanger 1 (NCX1)-deficient mice, which lack a normal heartbeat and blood circulation, have significantly less microglia in the embryonic brain (Ginhoux et al. 2010). Since loss of blood circulation could limit nutrient distribution and affect the normal physiology and metabolism of the embryo, other downstream events could be responsible for the microglial defects observed. Supporting this, *in vivo* imaging of larval zebrafish showed a circulation-independent mechanism for microglial invasion from brain-surrounding tissues into the parenchyma, although it is unclear if this migratory mechanism is specific to particular species (Jin et al. 2012). Interestingly, the fractalkine receptor CX3CR1 is required for the proper dispersion of macrophage precursors throughout mouse embryos. In mice that lack CX3CR1 expression, more precursor cells are stuck in the YS instead of colonizing the brain and other tissues. However, this defect is only temporary during embryonic stages (Kierdorf et al. 2013), which suggests that there must be other signals that attract microglia precursors to the CNS. Lysophosphatidylcholine (LPC) (J. Xu et al. 2016) or nucleotides (Casano, Albert, and Peri 2016) released by apoptotic neurons are suspected to be possible signals.

Microglia enter the brain rudiment *via* the leptomeninges and lateral ventricles by GD9.5 and distribute throughout the cortical wall from both directions at different speeds with varying rates of proliferation and maturation (see below), based on region and developmental stage (Arnoux et al. 2013; Ginhoux et al. 2010; Swinnen et al. 2013). They initially have amoeboid morphology and become ramified at GD14.5 (Kierdorf et al. 2013; Swinnen et al. 2013), when they are still highly proliferative, a state that lasts until the first postnatal week and

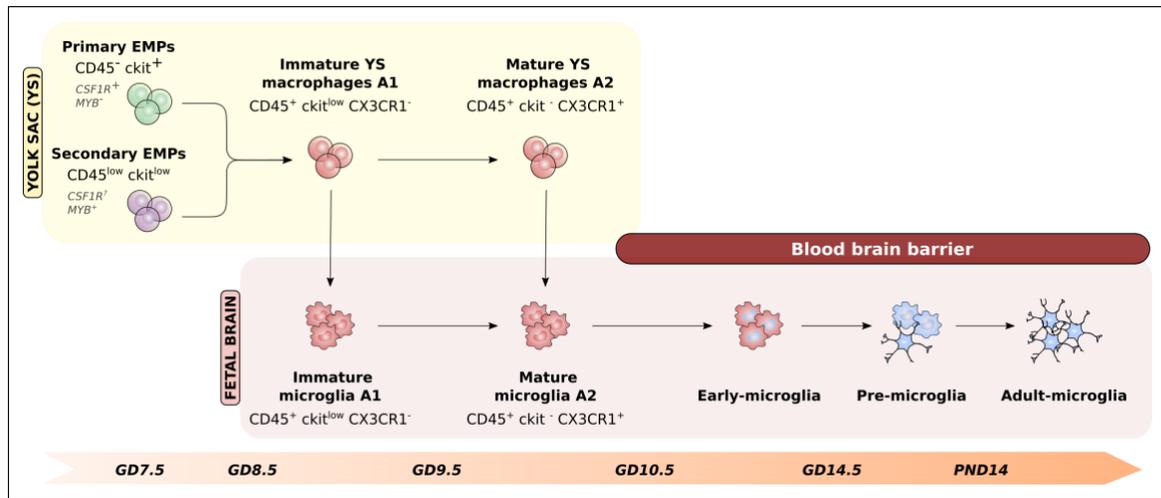
allows for growing coverage of the CNS (Bennett et al. 2016; Kierdorf et al. 2013). By postnatal day (PND)14.0, microglia are fully mature and express adult-signature genes (Bennett et al. 2016; Butovsky et al. 2014). Under normal conditions, this population of microglia cells maintains itself at constant densities (regionally specific), through tightly coupled local proliferation and apoptosis for nearly the entire lifespan (Askew et al. 2017).

### **- Microglial precursors differentiation -**

CSF1R signaling is known as essential for microglial development and maintenance (Chitu et al. 2016; Chitu and Stanley 2017). Indeed, during embryogenesis, YS macrophages depend on CSF1R signaling for their proliferation, differentiation and survival. In CSF1R-mutant mice, YS macrophages are absent and the subsequent tissues colonization by macrophages fails to occur (Ginhoux et al. 2010). Similarly, transient inhibition of CSF1R signaling pathway with a blocking anti-CSF1R antibody efficiently eliminates fetal macrophages, including developing microglia (Hoeffel et al. 2015). CSF1R signaling may induce myeloid cell differentiation by activating the myeloid lineage-determining transcription factor PU.1, which is broadly expressed by hematopoietic cells. Indeed, PU.1-mutant mice have reduced expression of CSF1R (Rosenbauer et al. 2004), suggesting that there is a feedback loop between CSF1R signalling and PU.1 regulation. Interestingly, PU.1-deficient mice lack microglia and all non-parenchymal macrophages (Goldmann et al. 2016; Schulz et al. 2012). As soon as the precursors emerge in the YS, once the myeloid lineage is determined by expression of PU.1 and CSF1R, microglial progenitors (EMPs) start to differentiate into YS macrophages. They first become CD45<sup>+</sup> ckit<sup>low</sup> CX3CR1<sup>-</sup> immature YS macrophages A1 and then become the more mature CD45<sup>+</sup> ckit<sup>-</sup> CX3CR1<sup>+</sup> mature YS macrophages A2 (Kierdorf et al. 2013). Of note, the transcription factor interferon regulatory factor 8 (IRF8) is required for maturation of the YS macrophage precursors. Indeed, these precursors undergo apoptosis in *Irf8*<sup>-/-</sup> mice, resulting in markedly reduced numbers of embryonic and adult microglia (Kierdorf et al. 2013). At GD10.5, macrophages in the brain will have further matured in comparison to the YS as evidenced by down regulation of ckit in immature A1 and mature A2 microglia cells (Kierdorf et al. 2013). A transcriptomic study further dissected microglial development from GD10.5 onwards into three discrete steps based on differential

genes regulation of microglia cells in the developing brain to acquire different functions (early-, pre- and adult microglia), with abrupt shifts occurring at approximately GD14.5 and PND14 (Bennett et al. 2016; Matcovitch-Natan et al. 2016) (Figure I.2).

**Figure I.2. Migration and differentiation of microglia.**



**Figure I.2.** As soon as they arise from hematopoiesis sites, at GD7.5, primary and secondary erythromyeloid precursors (EMPs) start differentiating into yolk sac (YS) macrophages. These immature YS macrophages A1 become mature YS macrophages A2. At GD10.5 head macrophages have matured into either immature microglia A1 or mature microglia A2. From GD10.5 to PND14, microglia precursors go through early- and pre-microglia stages to mature into adult microglia. Adapted from (Kierdorf et al. 2013; Q. Li and Barres 2018; Matcovitch-Natan et al. 2016).

## I.2. The role of microglia in CNS development

### I.2.1. Patterning the developing CNS

Microglia are the resident brain macrophages and they have been traditionally studied as orchestrators of the brain immune response during infection, disease or maintenance of brain homeostasis. In this context, microglia are defined as “professional” phagocytes which are

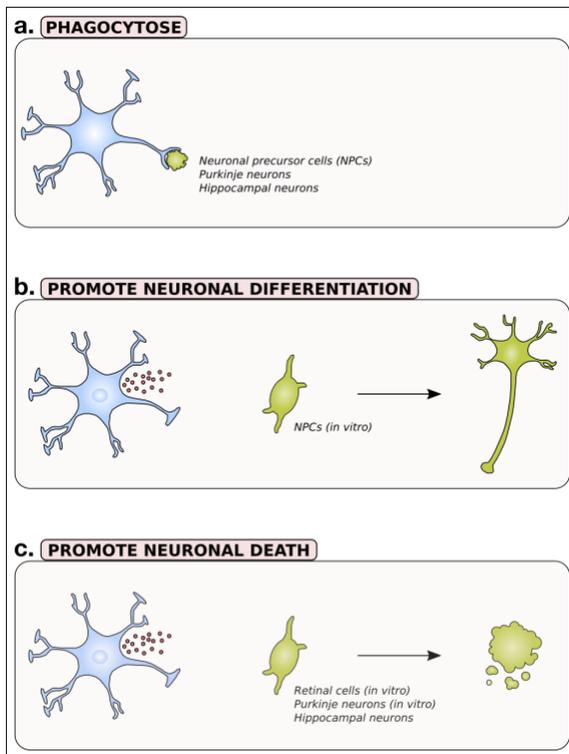
rapidly recruited to sites of damage where they engulf or phagocytose debris as well as unwanted and dying cells (Gehrmann, Matsumoto, and Kreutzberg 1995; Neumann, Kotter, and Franklin 2009). They also have the capacity to release a number of substances (cytokines, chemokines, nitric oxide, and reactive oxygen species), that can have both detrimental and beneficial effects on the surrounding cells (Czeh, Gressens, and Kaindl 2011). Interestingly, recent research has begun to explore the role of microglia in the healthy brain and how microglial phagocytic and signaling functions may help patterning the developing CNS.

The development of the brain and immune system are now known to be highly synchronized (Andersen 2003) and microglia are a critical link between these two processes. At each step of brain development, from migration and proliferation of neuronal progenitors, programmed cell death (PCD), formation of synapses to establishment of neuronal circuits, microglia are crucial to these developmental processes. Emerging data are suggesting new and fundamental roles for microglia in the control of proliferation and differentiation of neuron progenitor cells (NPCs). Indeed, the ability of microglia to **phagocytose** appears important in regulating the size of the NPCs pool. It has been discovered that, in primates and in rats, microglia contact, engulf and phagocytose neural precursors, including live and proliferating progenitors from GD17.0 to PND6 (Cunningham, Martinez-Cerdeno, and Noctor 2013). Similarly, another study also showed that microglia with phagocytic features were associated with decreased number of NPCs at GD17.5 (Tronnes et al. 2015). During cerebellar development, microglia actively promote engulfment-mediated Purkinje neuron death, by producing reactive oxygen species (ROS) as shown in sections of cerebellum from PND3 mice (Marín-Teva et al. 2004). During development, 50% of all neurons are subject to PCD (Yeo and Gautier 2004), a process that is critical for the establishment of a definitive pattern of neuronal connections. Microglia respond to PCD by phagocytosis of dead or dying neurons and its associated debris. Indeed, microglia migrate and significantly expand at different regions of the CNS at different times, usually right before or during the peak of PCD (Dalmau et al. 2003). This results in an overall high efficiency of microglia-removing apoptotic cells: microglia phagocytose 97% of apoptotic cells in the fimbria (embryonic day GD18.0), 88% and 100% in the hippocampus (GD16.0 and PND0 respectively), 18% and 86% in the cerebral cortex (GD16.0 and PND0 respectively), and 93% in the dentate gyrus

(PND0) (Dalmau et al. 2003) (Figure I.3a & I.5).

Moreover, microglia appear to influence events associated with **neuronal differentiation** during development. It has been demonstrated in culture that neurogenesis of embryonic cortical cells is enhanced by microglia (Aarum et al. 2003). The exogenous addition of microglia or conditioned medium from microglial cell culture promotes the differentiation of basal forebrain progenitors into cholinergic neurons (Jonakait et al. 1996, 2000). Similarly, Antony and colleagues suggested that microglia are an important component of embryonic NPCs differentiation in the cortex (Antony et al. 2011). Finally, pharmacological suppression of microglia activation significantly inhibits neurogenesis in the forebrain subventricular zone of the rat from P1 to P10, supporting the role of microglia in neuronal differentiation (Figure I.3b & I.5).

Microglia can also influence CNS patterning in opposite ways: they **promote neuronal death**. Indeed, microglia actively mediate cell death through soluble molecules. Historically, the first evidence for this role was found in the peripheral nervous system studying macrophages (analogous to CNS microglia). In cases of macrophage depletion, entire populations of ocular cells persisted for weeks after their usual time of death (Lang and Bishop 1993). Since this discovery, in addition to explants and slice studies (Frade and Barde 1998; Marín-Teva et al. 2004), *in vivo* evidence for microglia's role in directing cell death has emerged (Wakselman et al. 2008) (Figure I.3c & I.5).



**Figure I.3. Microglia role in patterning the developing CNS.**

**Figure I.3.** During CNS development, microglia (a) phagocytose neuron progenitor cells (NPCs), (b) promote neuronal differentiation of NPCs and (c) promote neuronal death. Adapted from (Q. Li and Barres 2018).

### ***1.2.2. Wiring the developing CNS***

In the postnatal brain, synaptic connections are formed in excess and must be remodeled to obtain an organized and efficient synaptic network, characteristic of mature organisms. Developmental synapse elimination (synaptic pruning) is a critical part of this remodeling. During development, neurons upregulate the expression of the chemokine fractalkine, CX3CL1 (Mody et al. 2001), and its receptor is exclusively expressed by microglia in the CNS (Harrison et al. 1998; Jung et al. 2000). This evidence led researchers to investigate the ability of microglia to **prune synapses** during development, to control synapse numbers. In 2011, a seminal study by Paolicelli and colleagues showed that microglia engulf synaptic material, thus play a major role in synaptic pruning during postnatal development (Paolicelli et al. 2011). Moreover, in mice lacking CX3CR1, microglia numbers were transiently reduced in the developing brain and synaptic pruning was delayed resulting in an excess of dendritic spines at PND15 (Paolicelli et al. 2011). Parallel studies on the postnatal retinogeniculate system identified the classic complement cascade as another underlying

mechanism in the process of synaptic pruning (Schafer et al. 2012). Indeed, complement component 3 (C3) is present at synapses during the early postnatal period and is required for normal developmental remodelling of retinogeniculate axons (Stevens et al. 2007). Given that microglia are the only known resident brain cells expressing the C3 receptor (CR3), the authors showed that C3-CR3 interactions allow the recruitment of microglia to retinogeniculate axons (Figure I.4a & I.5).

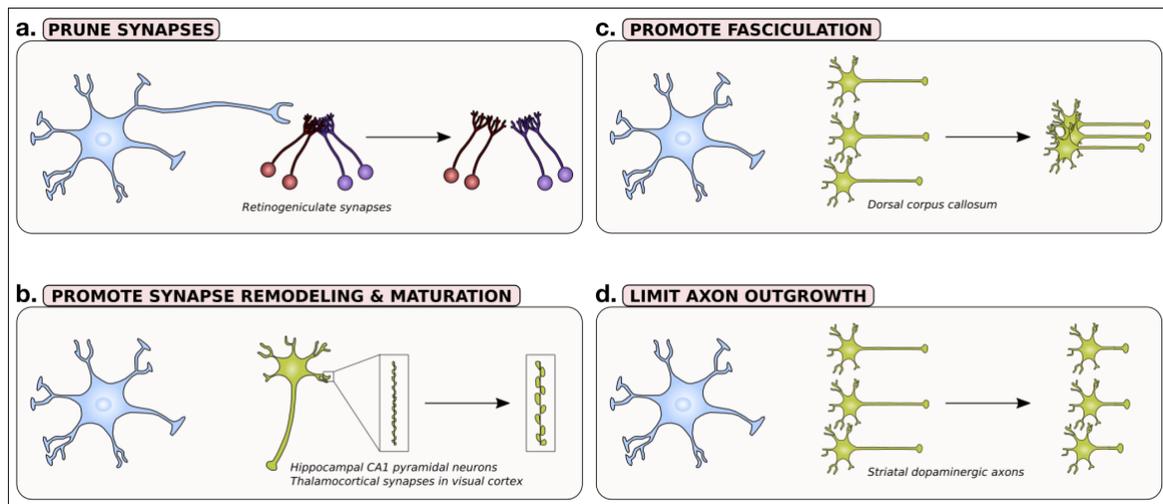
Mounting evidence has now shown that, during development, microglia are actively involved in **synapse remodeling and maturation** (Hoshiko et al. 2012; Paolicelli et al. 2011; Schafer et al. 2012). In CX3CR1 mutant animals, the dendritic spine density of hippocampal CA1 neurons transiently increases at PND15. This phenotype is associated with a temporary reduction in microglial cell numbers and with an accumulation of immature synapses, leading to decreased functional connectivity across brain regions and an autism-like behavioural phenotype (Paolicelli et al. 2011; Zhan et al. 2014). Moreover, it has been shown that microglial signalling is required for synaptic plasticity during the critical period of visual development (Sipe et al. 2016; Tremblay, Lowery, and Majewska 2010) (Figure I.4b & I.5).

In addition, microglia are closely associated with white-matter tracts during development, where they help to control axon **fasciculation** in the dorsal corpus callosum at GD17.5 (Pont-Lezica et al. 2014), thus promoting axons that travel in the same direction to adhere together to form a tight bundle (Figure I.4c & I.5).

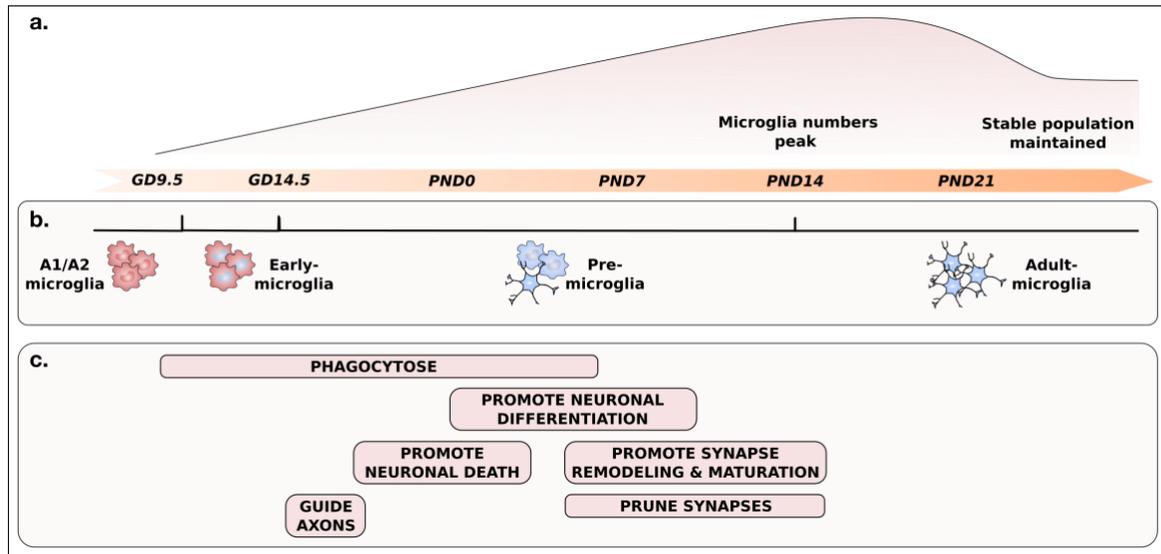
Microglia are also key players in the control of the wiring of forebrain circuits since they **limit axon outgrowth**. Microglial cells associated with axon fibers acquire a very distinct morphology and will line up parallel to the fibers, suggesting specific function of microglia in neurite development (Cuadros et al. 1993; Dalmau et al. 1997, 1998). This role of microglia is also supported by work performed with microglia-conditioned medium and primary neuronal cultures. Indeed, microglia-conditioned medium increases neurite growth and complexity (Chamak, Morandi, and Mallat 1994; Zhang and Fedoroff 1996). Until recently, there was no *in vivo* evidence of a definitive role for microglia in the various aspects of axonal growth and guidance. However, Squarzoni and colleagues carefully examined the positioning of microglial cells within the brain during early prenatal period (Squarzoni et al.

2014). They showed that at GD14.5, microglia transiently associate with the extremities of dopaminergic axons when they enter the subpallium, as opposed to adjacent serotonergic or internal capsule fibers. Furthermore, microglia are largely excluded from the cortical plate until GD16.5, at which point the cortical plate is then progressively colonized (Squarzoni et al. 2014). Using multiple mouse models, including cell-depletion approaches and  $CX3CR1^{-/-}$  or  $CR3^{-/-}$  mutants, they found that perturbing microglial activity affects the outgrowth of dopaminergic axons in the forebrain and the positioning of subsets of neocortical interneurons (Squarzoni et al. 2014) (Figure I.4d & I.5).

**Figure I.4. Microglia role in wiring the developing CNS.**



**Figure I.4.** During CNS development, microglia (a) prune synapses, (b) promote synapse remodeling and maturation, (c) promote axon fasciculation and (d) limit axon outgrowth. Adapted from (Q. Li and Barres 2018).

**Figure I.5. Overview of microglia during development.**

**Figure I.5.** (a) Illustrates microglia number across development. Microglia colonize and proliferate in the developing brain starting at gestational day (GD)9.0. Microglia numbers peak at postnatal day (PND)14 following local proliferation. Microglia number then decreases to reach adult number at PND28. (b) Indicates the different phenotypes of microglia across development. (c) Summarize microglia roles in the developing CNS (presented in detail in section I.3.). Adapted from (Kierdorf et al. 2013; Q. Li and Barres 2018; Matcovitch-Natan et al. 2016).

## I.3. The role of microglia in the mature CNS

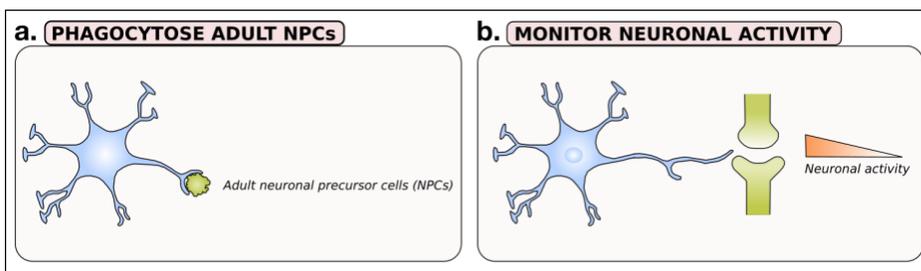
### I.3.1. Regulating mature CNS circuits

Emerging evidence suggests that microglia also play a key role in regulating established neural circuits in the mature CNS. While less widespread than the apoptosis that occurs during development, the process of PCD continues to some extent in mature animals. Indeed, adult neurogenesis occurs in the subgranular zone of the dentate gyrus in the hippocampus (Song et al. 2012). In this process, some of the newborn neurons are incorporated into circuits, whereas many are believed to undergo PCD (Song et al. 2012), and microglia seem

to **phagocytose these adult NPCs** (Sierra et al. 2010) (Figure I.6a).

In the optic tectum of larval zebrafish, it was shown that microglia preferentially contact neurons with higher levels of activity and that this activity was attenuated by the interaction (Y. Li et al. 2012). Moreover, they appear to be able to influence excitatory synaptic transmission by the release of modulatory factors like ATP (Pascual et al. 2012). Consequently, Pfeiffer and colleagues hypothesized and provided evidence that microglia **monitor neuronal activity** and contribute to activity-dependent synaptic plasticity in the healthy adult brain. Indeed, during hippocampal long-term potentiation (LTP), microglia alter their morphological dynamics by increasing the number of their processes and by prolonging their physical contacts with dendritic spines (Pfeiffer, Avignone, and Nägerl 2016) (Figure I.6b).

**Figure I.6. Microglia role in mature CNS.**



**Figure I.6.** In the mature CNS, microglia (a) phagocytose adult neuron progenitor cells (NPCs) and (b) monitor neuronal activity. Adapted from (Q. Li and Barres 2018).

### ***1.3.2. Surveilling the CNS***

Ramified morphology and the sparse expression of molecules associated with macrophage function in microglia of the healthy adult CNS have been associated with a “resting” phenotype. However, resting microglia are not dormant. Based on time-lapse recording in mice, it is estimated that in normal condition, microglia can scan the entire brain parenchyma every few hours. Indeed, microglia continuously extend and retract their fine processes in the healthy brain (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005).

Neighboring microglial cells take turns scanning shared regions, guaranteeing exhaustive screening while avoiding contact (Davalos et al. 2005, 2012). This process movement, termed “surveillance” of the brain, allow neurobiological (see above) and immunocentric functions throughout adulthood (Lenz and Nelson 2018). In light of such activity, the suitability of the term “resting” microglia is challenged. Indeed, if such surveillance is an activity that involves constant morphological alteration as well as continuous biochemical sensing and interpretation of environmental cues, the term ‘resting’ would miss an important constitutive function. The “resting” state may actually reflect a defined mode of an active cell and should be considered as a “surveying” state.

### ***1.3.3. Mediating CNS immune response***

Many molecules and conditions can trigger a transformation of resting (or surveying) microglia to activated (alerted or reactive) states. These have in common that they indicate a threat to the structural and functional integrity of the CNS either by their loss or by their appearance.

Microglia are equipped with a group of surface receptors which trigger signals in microglia under “resting” conditions. Inhibitory receptors like CX3CR1, CD200R and CSF1R expressed at the surface of microglia have their ligands (respectively CX3CL1, CD200 and CSF1 / CD34) on the surface of healthy neurons and/or astrocytes (Biber et al. 2007; Chitu and Stanley 2006; Hoek et al. 2016; Jung et al. 2000). A loss of these exogenous signals indicates a loss of neuronal/astroglial integrity, subsequently activating microglia, hence the name of “inhibitory” “off” signaling (Kierdorf and Prinz 2013).

Microglial responsiveness can also be triggered by the sudden appearance of factors that are not usually seen (or are not seen at critical concentrations) that are sensed by an array of receptors (Block, Zecca, and Hong 2007; Rossum et al. 2004). For example, the families of pattern recognition receptors, such as Toll-like receptors (TLRs), detect and differentiate viral, bacterial and fungal structures (Trinchieri and Sher 2007). Microglial responses are thereby triggered by induced receptor signaling.

**- M1 polarization -**

“Polarization” refers to perturbation of microglia with exogenous agents to achieve a particular phenotype and is evident by the presence of one or more molecular markers and a shift in molecular profile. Resident microglia become polarized toward a pro-inflammatory (M1) phenotype upon exposure to pro-inflammatory cytokines interferon gamma (IFN $\gamma$ ), TNF $\alpha$  and cellular or bacterial debris. This action is the first line of defence of the innate immune system and is geared to kill the offending foreign pathogen and polarize T-cells to mount an adaptive immune response. In many experimental models, the M1 response is characterized following exposure to bacterial-derived products such as, lipopolysaccharide (LPS) or signals associated with infection such as IFN $\gamma$  (Martinez and Gordon 2014). A similar but sterile inflammatory response can occur in the absence of microorganisms, often as a result of trauma, ischemia-reperfusion injury, or chemical exposure (Chen and Nuñez 2010; McPherson, Merrick, and Harry 2014). Microglia respond by producing M1 associated factors such as, pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-12, IL-23, TNF $\alpha$ ), chemokines, redox molecules (NOX, PHOX, iNOS), scavenger receptors [macrophage receptor with collagenous structure (MARCO), co-stimulatory proteins (CD40)], and major histocompatibility complex-II (MHC-II) (Boche, Perry, and Nicoll 2013; Colton and Wilcock 2010; Hanisch and Kettenmann 2007; Henkel et al. 2009; Ransohoff and Perry 2009; Varnum and Ikezu 2012).

**- M2 polarization state -**

In contrast with the M1 phenotype, the M2 phenotype is more heterogeneous, there is not one set description or classification of M2 cells. Division of M2 cells is based on observations that stimulation with various cytokines yields different sets of receptor profiles, cytokine production, chemokine secretion, and function (Mantovani, Bonecchi, and Locati 2006). However, these cells have one feature in common that places them all in the M2 classification: express mediators or receptors with the capacity to downregulate, repair, or protect the body from inflammation (Varin and Gordon 2009).

The original alternatively activated macrophage was classified based on expression of the

mannose receptor (Stein 1992). Since, other markers have been identified as “M2” specific [e.g. the enzyme arginase 1 (Arg1) (Munder et al. 1999)]. Another way to classify the function and phenotype of M2 cells is based on the cytokines that induce them. The prototypical cytokine used to first induce alternative activation was IL-4 (Stein 1992). Both IL-4 and the closely related cytokine IL-13 signal through IL-4R $\alpha$  to induce a host of downstream processes that lead to potent anti-inflammatory functions, such as Arg1 upregulation and production of scavenger receptors for phagocytosis (Gordon and Taylor 2005; Sica and Mantovani 2012). This type of activation has been classified as “M2a”. The main function of this response appears to be suppression of inflammation. A second state of alternative activation is based on macrophages exposed to IL-10, glucocorticoids, or TGF- $\beta$ . This phenotype is classified as “M2c” and appear to be involved in tissue remodeling and matrix deposition after inflammation has been downregulated (Sica and Mantovani 2012). A third sub-class of M2 activation has been observed following exposure to immune complexes and stimulation of TLR and is termed “M2b” (Sica and Mantovani 2012). It has been suggested that these cells might be potential regulators or initiators of the M2 response in general (Edwards et al. 2006).

An important consideration regarding M2 phenotypes is that these states were typically elucidated *in vitro* following exposure to stimuli. This does not represent the complex environmental milieu seen *in vivo*. Therefore, some investigators have cautioned against this classification into distinct subtypes and instead propose that M2 cells should be viewed as a spectrum of phenotypes (Mosser and Edwards 2010).

### **- Polarization transition -**

As previously stated, M1 microglial activation aims to promote killing of any invading organism and to remove dead cells to ‘clean’ the damaged area. To efficiently repair the damage, this pro-inflammatory response is then shifted to an anti-inflammatory (M2) state where debris clearance, extracellular matrix deposition, and angiogenesis are promoted.

This transition can be caused by actual reprogramming of macrophages and/or replacement of M1 cells with recruited monocytes that differentiate into M2 under the influence of the newly established environment (Das et al. 2015). Moreover, in case of inflammation-induced

neutrophil infiltration, the clearance of these apoptotic neutrophils has been shown to prompt a switch from a pro- to an anti-inflammatory macrophage phenotype (Fadok et al. 1998; Michlewska et al. 2009). While M2 macrophages are highly plastic and can be easily polarized in an M2 state, the M1-to-M2 transition is more complex (Van den Bossche et al. 2016; Davis et al. 2013).

## **I.4. Heterogeneity of microglia**

### *I.4.1. Heterogeneity of microglia throughout the brain*

The CNS is composed of many different regions with unique functional specializations. Knowing the numerous functions of microglia in the CNS, it is logical to expect heterogeneity in the distribution of microglia. Such regional heterogeneity was first highlighted in 1990 by Lawson et al., showing that microglial density is higher in the hippocampus and thalamus compared with the cortex and cerebellum (Lawson et al. 1990). They also analyzed the morphological features of microglia prevalent in different adult brain regions and showed that their degree of ramification change depending on where they reside (Lawson et al. 1990). For instance, the periventricular white matter (the corpus callosum) has a high density of amoeboid microglia. Under steady-state conditions, amoeboid microglia gradually become ramified microglia with advancing age (Ling and Wong 1993). Reciprocally, as discussed before, ramified microglia can retract their ramifications and acquire an amoeboid shape in case of activation by infection for example. Interestingly, transcriptomic analysis of amoeboid and ramified microglia showed that under steady-state conditions, amoeboid microglia produce equivalent levels of cytokines and chemokines to ramified microglia, but uniquely express genes involved in the cell cycle and apoptosis (Parakalan et al. 2012). Amoeboid microglia were also enriched in genes implicated in synaptic transmission. Since the work of Lawson et al., advanced microscopy tools have allowed scientists to study microglia morphology and cellular organization in finer detail. A recent study found that the volume and size of cell body and cytoplasmic regions of microglia differ along brain regions (Verdonk et al. 2016). Some brain regions are subdivided into substructures, further highlighting the complexity of brain organization. One example is the

basal ganglia where microglial density differs between the three sub-regions (De Biase et al. 2017). This heterogeneity in microglia distribution within the basal ganglia is associated with specific membrane properties, as electrophysiological recordings demonstrated that microglia have different hyperpolarized resting potential depending on the sub-region. Finally, a transcriptomic analysis of microglia present in these substructures showed a heterogeneity in expression level of genes suggesting specific metabolic activities in these microglial subpopulations (De Biase et al. 2017). Other gene expression analyses have identified differences in microglial expression profiles between brain regions under normal physiological conditions (Doorn et al. 2015; de Haas, Boddeke, and Biber 2008). A recent study identified variability in the tightly regulated processes underlying microglial maintenance (Tay et al. 2017). Indeed, they estimated that turn over in full of microglial population can take from 8 months up to 41 months depending on the brain region.

#### ***1.4.2. Heterogeneity of microglia across sex***

In addition to regional heterogeneity, microglia present sex-related differences. A growing body of research documents sex differences in microglia during development and more precisely during the critical period for sexual differentiation. One of the first studies to compare male and female microglia found that females have more microglia in the hypothalamus, amygdala, and hippocampus shortly after the prenatal testosterone surge while in the early postnatal period, males have more microglia than females (Schwarz, Sholar, and Bilbo 2012). Females also have more microglia in the somatosensory area throughout perinatal development (Thion et al. 2018). Others showed that males have more microglia than females overall and more amoeboid shaped microglia in the developing preoptic area (POA) and these differences are induced by exposure to estradiol (Lenz et al. 2013). Indeed, treating females neonatally with estradiol masculinize microglia in the POA (Lenz et al., 2013). Microglial phagocytosis in the hippocampus also presents sex difference. Microglia phagocytose more cells in the neonatal female hippocampus than in the neonatal male hippocampus, and this sex difference is regulated by hormones. Indeed, estradiol treatment of females reduces phagocytic microglia to male-typical numbers (Nelson, Warden, and Lenz 2017). Concurrently, females also had higher expression of several

phagocytic pathway genes in the hippocampus compared to males (Nelson, Warden, and Lenz 2017). In line with these findings, a recent study has demonstrated that, in the neonatal hippocampus, female microglia express more CD68 (a lysosomal marker used to indicate phagocytic capacity) in the second postnatal week (Weinhard et al. 2018). These effects could be modulated by sex hormones, considering that isolated microglial cells express both estrogen and progesterone receptors (Sierra et al. 2008). On the transcriptional level, while the microglial transcriptome is similar during development, sexual dimorphism increases in adulthood (with female microglia in a more immune-activated state) (Hanamsagar and Bilbo 2016; Thion et al. 2018). However, it remains unknown whether these differences in maturity alters any normal developmental processes, such as cell genesis, death or synaptic patterning, between the sexes.

Altogether, these data suggest that adult microglia do not form a homogeneous population under steady-state conditions but behave differently depending on their environment. A disturbance of this highly specific heterogeneity might result in serious long-term consequences arising from the disruption of brain development and or function.

*The above is a general review of our current knowledge of microglial development and function and reflects the heterogeneity of these cells as well as their wide-ranging task in the developing and adult brains. The inherent sensitivity of these cells to changes in their immediate environment underlies their ability to perform their task in a highly efficient manner. The efficiency of microglia is a result of a highly coordinated process, which if interrupted makes the cells vulnerable to multiple detrimental exogenous triggers. Perhaps one of the best-known situations where interruption of microglial function results in serious long-term consequences is during critical time points during gestation when the pregnant mother is exposed to an environmental challenge that results in activating the maternal immune system and subsequently the microglia in the fetal brain. Much of our knowledge of this interaction has come from numerous animal models developed to investigate the impact of maternal environment challenge on the developing fetal brain and the subsequent behavioural changes in the offspring.*

## **I.5. Maternal Immune Activation & neurodevelopmental disorders**

Extensive literature has demonstrated that maternal viral and bacterial infections during pregnancy are associated with an increased incidence of neurodevelopmental disorders including schizophrenia (SCZ) and autism spectrum disorder (ASD) in resulting offspring (Brown and Susser 2002; Cannon, Jones, and Murray 2002; Rapoport, Addington, and Frangou 2005) [see (Brown 2012) for extensive review]. This idea originally emanated from work first reported in the mid 1980s, when numerous groups demonstrated that maternal influenza during pregnancy is associated with an increased incidence of SCZ (Mednick et al. 1988; Mednick, Huttunen, and Machon 1994; Munk-Jorgensen and Ewald 2001; O’Callaghan et al. 1991; Takei 1996). The most convincing evidence to date for an association between maternal influenza and SCZ comes from work by Brown et al (Brown et al. 2004). Using archived maternal serum assayed for influenza antibodies to confirm infection, these authors demonstrated a 7-fold increased risk for SCZ associated with maternal influenza infection during the first trimester. Increased incidence of SCZ has also been associated with other viral infections during pregnancy including measles (Torrey, Rawlings, and Waldman 1988), rubella (Brown and Susser 2002), varicella-zoster and polio (Torrey, Rawlings, and Waldman 1988) and with bacterial infections during pregnancy, such as diphtheria and pneumonia (Watson et al. 1984), and other respiratory infections (Brown et al. 2000; A S Brown and Susser 2002). The wide variety of infectious agents involved suggests that factors common to the infectious process such as inflammation (*e.g.* elevations in cytokine levels), rather than one specific infectious agent, may be responsible for increases in SCZ.

Whilst the early work describing the association between maternal infection and neurodevelopmental disorders focussed on SCZ, there is now substantial evidence linking these maternal events with other disorders such as ASD and depression. Atopic autoimmune and allergic disorders have been associated with ASD and suggest a relationship between immune system dysfunctions and ASD pathogenesis (Keil et al. 2010; Pardo, Vargas, and Zimmerman 2005; G. Xu et al. 2014). Evidence of immune system dysregulation in ASD includes up-regulation of cytokines and their downstream signaling pathways in the CNS of

affected individuals (Vargas et al. 2005). Expression profiling in a small number of autistic and control brains revealed increased transcript levels of many immune related genes, including many components of the IL-1 $\beta$  and IL-6 pathways (Garbett et al. 2008; Voineagu et al. 2011). Moreover, increased levels of pro-inflammatory and decreased levels of anti-inflammatory cytokines measured in the peripheral blood plasma of individuals with ASD provide evidence of immune dysfunction (Ashwood et al. 2011; Estes and McAllister 2015; Masi et al. 2015). Finally, cytokine expression levels have been correlated with the clinical severity of ASD symptoms (Masi et al. 2015). Of note, it has been proposed that environmental factors may trigger immune activation as a risk factor for ASD. Pollutants, food additives and cosmetic ingredients may interact with genetic predispositions to increase the incidence of ASD partly through inflammatory pathways (Carter and Blizard 2016). Such environmental factors can induce an infection-free maternal immune activation (MIA) response that may contribute to ASD-like behavioural dysfunction and other neurodevelopmental defects [see (Bilbo et al. 2018) for review]. One of the other neurodevelopmental consequences is a predisposition to developing depression in offspring that have undergone inflammatory type stressor *in utero* [see (Murphy et al. 2017) for review]. Whilst substantial literature exists associating early (postnatal) life stress with the development of depression (Heim and Binder 2012), relatively little investigations have been conducted in relation with prenatal infections. This is in part due to the difficulties associated with performing epidemiological studies in populations that have been exposed to infectious agents in times such as during epidemics and in performing mechanistic type studies in humans. These challenges were in part responsible for the development of preclinical approaches involving animal models.

**- Animal models for investigating neurodevelopmental disorders -**

Perhaps the best-known experimental approach to investigate the impact of maternal infection/inflammation during pregnancy on the incidence of neurodevelopmental disorders in the offspring has been the maternal immune activation (MIA) approach using rodents [see (Boksa 2003; Boksa 2010) for reviews]. The maternal immune system can be activated in a number of ways that range from stress to diet and even environmental pollution. However,

the most potent activator remains exposure to immunogenic challenge. Systemic viral [Poly(I:C)] or bacterial LPS immunogens are part of the most common immunogens used to model maternal infection in animals. These immunogens are powerful inducers of inflammatory mediators (Ashdown, Dumont, et al. 2006; Ashdown, Poole, et al. 2006; Cui et al. 2009; Luheshi 1998) and treatment of pregnant animals (primarily rodents) with either has been shown to induce a substantial maternal immune response and to elicit changed behaviours in the offspring that are associated with ASD and SCZ in humans [see (Boksa 2010; Kneeland and Fatemi 2013; Meyer 2013, 2014; Patterson 2009; Solek et al. 2018) for reviews]. These behaviours include **increased anxiety** in the adult animal (Babri, Doosti, and Salari 2014; Hsiao et al. 2013; Hsueh et al. 2017; Meyer 2006; Meyer et al. 2005; da Silveira et al. 2017) and **decreased social interest**, often observed in MIA animals (Aavani et al. 2015; Bitanirwe et al. 2010; Fernández de Cossío et al. 2017; Hao et al. 2010; Onore et al. 2014). Prepulse inhibition (PPI) is used as a measure of the ability of the brain to filter out extraneous information and **deficits in PPI** have been observed in schizophrenia patients. Indeed, substantial number of MIA type studies have resulted in PPI deficits in animal models (Basta-Kaim et al. 2015; Fortier, Luheshi, and Boksa 2007; Howland, Cazakoff, and Zhang 2012; Makinodan et al. 2008; Meehan et al. 2017; Meyer et al. 2008; Meyer et al. 2005; Vorhees et al. 2012, 2015; Vuillermot, Feldon, and Meyer 2010; Wischhof et al. 2015; Wolff and Bilkey 2010), while some conditions produced no significant effect on PPI (Van Den Eynde et al. 2014; Giovanoli et al. 2015; Missault et al. 2014). **Impairments in learning and memory**, have also been described in schizophrenia and ASD patients (Goh and Peterson 2012; Paulsen et al. 1995). Several behavioural tests are used in rodents to assess similar aspects of cognitive function (Young et al. 2009), and a vast majority of MIA studies demonstrated significant learning and memory deficits in rodents (Batinić et al. 2016; Howland, Cazakoff, and Zhang 2012; Labrousse et al. 2018; Luchicchi et al. 2016; Meehan et al. 2017; Yin et al. 2013). Only one study using LPS-induced MIA in rats detected no difference in spatial learning (Yin et al. 2015).

One of the core symptoms of ASD is **repetitive behaviours**, which include repetitive body movements, repetitive motions with objects, rituals and unusual or very narrow restricted interests. These behaviours can be observed in rodents through evaluation of repetitive self-

grooming and compulsive marble-burying behaviour (Fernández de Cossío et al. 2017; Kirsten and Bernardi 2017; Silverman et al. 2010). Another important assessment is ultrasonic vocalizations (USVs) in isolated pups that can be affected in a number of complex ways, which may indicate **altered emotional or social behaviour and communication** (Moy and Nadler 2008). There is a great deal of variability in the effects of MIA on USVs. For example, whilst we (Fernández de Cossío et al. 2017) measured prenatal LPS-induced shorter USVs but unaffected number of USVs, others measured reduced (Baharnoori, Bhardwaj, and Srivastava 2012; Malkova et al. 2012) or increased (Schwartz et al. 2013; Yee et al. 2012) number of USVs. It has been suggested that these differences might be due in part to the different methodologies used to record the USVs (Hahn and Lavooy 2005). Finally, an enhanced locomotor activity reaction to low doses of amphetamine is interpreted as an indication of a functional **imbalance in dopaminergic transmission** underlying a subset of schizophrenia symptoms (Schmidt et al. 2010). Most often, MIA induces an increase in the amphetamine-induced locomotor activity (Batinić et al. 2016, 2017; Howland, Cazakoff, and Zhang 2012; Meyer et al. 2008; Missault et al. 2014; Vuillermot, Feldon, and Meyer 2010; Zager, Mennecier, and Palermo-Neto 2012), but as with other behavioural assays, some MIA studies report no change in this behaviour (Holloway et al. 2013; Meehan et al. 2017). It is clear from these and other similar studies that the behaviours exhibited by the offspring of infected mothers is significantly altered and whilst human disorders such as depression, SCZ or ASD cannot be truly modeled in animals, the behavioural paradigms tested in these types of investigations acts as a good indicator of dysfunctional brain processes and provide a good experimental approach for identifying mechanisms underlying psychiatric disorders in humans.

## **I.6. Maternal Immune Activation & microglia**

Whilst inflammation has been implicated in the triggering of neurodevelopmental disorders, until recently the evidence was limited to assessments of changes in inflammatory mediators or the gross histochemical studies showing morphological changes in glia in post mortem brain and brains from MIA type models. The implication of microglia in early

neurodevelopment has resulted in a seismic shift in the approaches used to investigate the mechanisms involved. However, the numerous studies using MIA to investigate microglial alterations produced significantly divergent results which are due mainly to variability in outcome assessment. One example is microglial activation that can be measured by an estimation of the number/density of microglia or by the assessment of different microglial markers. In general, direct comparison of different studies is nearly impossible since brain region and time points investigated differ. Numerous other factors also need to be considered including the immune stimulant used, frequency of immune challenges, and route of administration that could all contribute to the observed inconsistencies in microglia phenotypes in the MIA model. The other major factor of divergence is likely to be the gestational time point of immune activation.

#### ***1.6.1. Effects of Maternal Immune Activation on embryonic microglia***

The induction of inflammatory cytokines in the embryonic brain by MIA within few hours (Ashdown, Dumont, et al. 2006; Labrousse et al. 2018) suggested that MIA could alter microglia activation states during prenatal development. Moreover, some genes expression was recently measured in embryonic microglia as early as GD10.5 (Thion et al. 2018), adding support to these findings.

Results from MIA-induced embryonic microglia activation are heterogeneous. For example, microglial density in MIA offspring is either unchanged or increased depending on the study. Increased microglia density and altered microglia morphology were measured in the somatosensory cortex 24 hours after MIA at gestational day (GD)17.5 (Tronnes et al. 2015), whilst no evidence of altered microglia density was detected in the fetal cortex and hippocampus 24 hours after maternal injection of Poly(I:C) at GD11.5 (Smolders et al. 2015). Again, direct comparison between these two studies is problematic due to the difference in MIA paradigm and gestational window of MIA induction. Other studies detected no changes in fetal microglia density or morphology 48 to 72 hours post-MIA induction (Cunningham, Martinez-Cerdeno, and Noctor 2013; Pont-Lezica et al. 2014; Pratt et al. 2013; Smolders et al. 2015).

Interestingly, while some studies did not measure alterations in microglia density or

morphology following MIA, they measured altered gene and protein expression of inflammatory mediators in embryonic brain or microglia (Cunningham, Martinez-Cerdeno, and Noctor 2013; O’Loughlin et al. 2017; Pont-Lezica et al. 2014; Pratt et al. 2013; Schaafsma et al. 2017). Pont-Lezica et al., used genome-wide gene expression profiling of isolated embryonic microglia and found a differential gene expression of 3906 genes after MIA (Pont-Lezica et al. 2014). More specifically, several immune genes such as IL-1 $\beta$ , C–C motif Chemokine ligand 4 (CCL4) and Nuclear factor (NF)- $\kappa$ B were upregulated while microglial genes involved in neuritogenesis were downregulated (Pont-Lezica et al. 2014). Immunohistochemical analyses on embryonic brain slices from rats exposed to LPS of microglial activation state markers showed that the number of microglia expressing Arg1 (M2 marker) was not changed but the proportion of microglia expressing iNOS, IL-1 $\beta$  or IL-1ra (M1 markers) was increased upon LPS administration (Cunningham, Martinez-Cerdeno, and Noctor 2013). Similarly, repeated prenatal LPS administration in mice increased mRNA levels for IL-1 $\beta$ , IL-6 and TNF $\alpha$  in isolated microglia 3hours after the last LPS injection (Pratt et al. 2013; Schaafsma et al. 2017). In contrast to these findings, no change in microglial protein levels of inflammatory markers was detected in the hippocampus and cortex of embryos at different developmental stages following a single Poly(I:C) exposure at GD11.5 or repeated immune stimulation at GD11.5 and GD15.5 (Smolders et al. 2015). Taken together, definitive conclusions cannot be drawn from the current literature on MIA-induced microglial alterations. However, it is clear that embryonic microglia are able to sense and respond to MIA, causing long-term changes in their transcriptomic signature (Mattei et al. 2017).

### ***1.6.2. Effects of Maternal Immune Activation on postnatal microglia***

#### ***- Microglia number/density -***

Since upon detection of an insult, microglia migrate to the site of injury/infection and/or proliferate (Streit, Walter, and Pennell 1999), microglial activation is often measured in relation to their number/density in specific brain regions of interest. Among studies measuring microglial density in brains of adult MIA offspring (>PND60), some reports showed increased microglial density (Van Den Eynde et al. 2014; Girard et al. 2010) while

the vast majority reported no change (Buschert et al. 2016; Corradini et al. 2018; Garay et al. 2013; Giovanoli et al. 2015, 2016; Hsiao et al. 2012; Hsueh et al. 2017; Hui et al. 2018; Missault et al. 2014; Mouihate 2016; Paylor et al. 2016). Similar to the findings in adult brains, measurements in younger adolescent offspring provides more evidence for unchanged microglial densities (Garay et al. 2013; Giovanoli et al. 2015, 2016; Hsueh et al. 2017) in comparison to increased density (Girard et al. 2010). Interestingly, some studies showed that the density of microglia can vary depending on the age and sex of the animal, as well as the brain region investigated (Hadar et al. 2017; Juckel et al. 2011; Manitz et al. 2013; Mattei et al. 2014).

These results suggest that MIA only sporadically induces microgliosis in certain brain regions. The biological function of increased numbers of microglia in specific brain regions of MIA exposed offspring and its consequences on brain formation and maturation has yet to be identified.

#### **- *Microglia morphology* -**

A second parameter often used to assess “microglia activation” is the cell’s morphology. Ramified microglia for example represent a “resting” state while amoeboid forms are interpreted as “activated” state (Hanisch and Kettenmann 2007). Similar to the microglia density analyses, microglial morphology in MIA is variable. The findings vary from decreased numbers of processes - indicative of the amoeboid state - (Corradini et al. 2018; Hui et al. 2018; Juckel et al. 2011; Missault et al. 2014; O’Loughlin et al. 2017), to increased ramification (Gumusoglu et al. 2017) while others detected no changes in microglia morphology in MIA adult offspring (Garay et al. 2013; Giovanoli et al. 2015, 2016; Hsueh et al. 2017; Missault et al. 2014; Mouihate 2016).

#### **- *Microglia activation state* -**

The analysis of a single aspect in the continuum of microglia activation states is insufficient to capture subtle changes in microglia reactivity, especially in the MIA model, where subtle changes in microglia activity have been detected using highly sensitive approaches including microglia-specific mRNA sequencing (Matcovitch-Natan et al. 2016). That is why a widely

used approach is to complement microglia density and morphological analyses with assessing the expression of microglial surface markers as well as cytokine and chemokine levels. Amongst the most widely used microglia markers are: CD68 (a lysosomal-associated membrane glycoprotein known to be upregulated in M1 states of microglia), CD11b (subunit of the complement receptor 3 complex), MHCII, CX3CR1, CD200R (inhibitory cell surface receptor), and Iba1 as well as various different pro- (IL-1 $\beta$ , IL-6, TNF $\alpha$ ) and anti-inflammatory (IL-5, IL-10) cytokine and chemokine levels.

Again, the findings in the expression pattern of microglia markers in MIA models are diverse. For example, while studies found no MIA-induced change in the expression of CD68, CD11 or Iba1 (Corradini et al. 2018; Giovanoli et al. 2016; Hsueh et al. 2017; Krstic et al. 2012; Manitz et al. 2013; Willi et al. 2013) others found changes in the expression of these same markers (either increased or decreased) (Borrell et al. 2002; Van Den Eynde et al. 2014; Manitz et al. 2016; Mattei et al. 2017; Mattei et al. 2014). Two recent studies measured changes in the expression of the mitochondrial translocator protein (TSPO) in the brains of MIA offspring, similar to *in vivo* PET imaging studies in humans (Mattei et al. 2017; Notter et al. 2018) and both found opposite results. The reason for this could be the difference in brain region or in the MIA protocol.

Besides microglia surface markers, a number of studies have assessed the expression of pro- and anti-inflammatory cytokines in MIA offspring. Although not specific for microglia, several studies determined cytokine levels in brain homogenates in an attempt to give an idea about the inflammatory status of the brain. Once again, findings are divergent (Garay et al. 2013; Gilmore, Jarskog, and Vadlamudi 2005; Giovanoli et al. 2015, 2016; Krstic et al. 2012; Mattei et al. 2017; Notter et al. 2018; O’Loughlin et al. 2017). Of note, cytokines are not only produced by microglia, but also by other parenchymal cell types (such as astrocytes and neurons). Only few studies measured cytokine levels in isolated microglia from adult MIA offspring. Strikingly, although the protocols used to induce MIA and the age and brain region analyzed are the same, one showed increased levels of IL-1 $\beta$  mRNA (Mattei et al. 2014), while the other found no change in IL-1 $\beta$  mRNA levels (Hadar et al. 2017).

Overall, such variability in the current literature does not allow for clear conclusion on MIA-induced microglial alterations. However, it appears that MIA has consequences on microglia

during development and at adulthood. Early embryonic insult might deviate the phenotypical track of microglia, which in turn could induce neuronal and behavioural alterations.

## **I.7. Hypothesis**

This thesis addresses the hypothesis that exposure to a single inflammatory stimulus alters the normal microglial responses in a sexually dimorphic manner, in both adult and embryonic brains. These responses result in behavioural anomalies associated with brain disorders in humans, some of which have a neurodevelopmental origin.

## **I.8. Objectives**

The aims of the work presented in this thesis are:

### **1. Determine the role of microglia in mediating short-term LPS-induced inflammatory changes and depressive-like behaviour.**

The approach will involve the depletion of phagocytic microglia *via* an intra-hippocampal administration of liposomes containing dichloromethylene-bisphosphonate (clodronate), inducing apoptosis of phagocytic microglia, before the administration of LPS. The inflammatory and behavioural outcomes will then be evaluated.

### **2. Determine the consequences of early prenatal administration of LPS on the offspring' microglia population and behaviour.**

This will be conducted comparing embryonic microglial population and a large panel of behaviours during the neonatal period and at adulthood in offspring from mothers having received prenatal administration of LPS or Saline as a control.

### **3. Investigate the mechanisms underlying the changes in microglia population and behaviour induced by prenatal LPS; and determine the implication of microglia in mediating long-term behavioural alterations induced by prenatal LPS.**

This will be conducted with correlative assessment of prenatal LPS-induced microglial and

behavioural alterations. A particular focus of these studies will be to identify the role of the pro-inflammatory cytokine IL-1 $\beta$  which is a major modulator of microglial activity.

**Chapter II:**  
**Hippocampal microglia depletion improves LPS-induced  
depressive-like behaviour and IDO expression**

## **II.1. Manuscript**

### **Title: Hippocampal microglia depletion improves LPS-induced depressive-like behaviour andIDO expression**

**Chloé Lacabanne<sup>1,2</sup>, Anouk Benmamar--Badel<sup>2</sup>, Giamal N. Luheshi<sup>2\*</sup> and Sophie Layé<sup>1\*</sup>**

<sup>1</sup> Nutrition et Neurobiologie Intégrée, UMR 1286, INRA, Université de Bordeaux,  
Bordeaux, France

<sup>2</sup> Douglas Hospital Research Centre, Department of Psychiatry, McGill University, Quebec,  
Canada H4H 1R3

\*Equal contribution

## Abstract

Microglial activation following an environmental inflammatory challenge, are known to trigger neurotoxic effects some of which have been associated with chronic brain disorders such as depression. Experimental animal studies using the bacterial cell wall product lipopolysaccharide (LPS), demonstrated that a single systemic injection of this immunogen induces depression-like behaviour in rodents through the release of pro-inflammatory cytokines in the brain. Some studies have associated these inflammatory changes in the brain to activation of microglial cells, which involves polarization of these cells between the M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes. In the current study, we investigated the specific contribution of these cells to the overall CNS inflammatory response in mice treated with an injection of LPS. For this, we depleted phagocytic M2-like microglia using liposome-mediated intracellular delivery of dichloromethylene-bisphosphonate (clodronate) injected in the CA3 region of the hippocampi prior to LPS. Hippocampal CA3 region is associated with changes in cognitive behaviour in depression as well as susceptibility to changes in inflammatory milieu. As expected, LPS induced a significant increase in microglia activation, which was abolished by the Clodronate treatment in the CA3 region. The analysis of the inflammatory mediators' expression revealed a significant increase in the pro-inflammatory cytokine interleukin (IL)-1 $\beta$  that was potentiated by the Clodronate administration while LPS-induced expression of the pro-inflammatory cytokine tumor necrosis factor (TNF) $\alpha$  and the anti-inflammatory cytokine IL-10 were significantly attenuated by liposome-mediated phagocytic microglial depletion. In addition, LPS induced an increase in the expression of the tryptophan degrading enzyme indoleamine-2,3-dioxygenase (IDO) used as a correlative measure of depression-like behaviour, that was attenuated by clodronate administration. Conversely, LPS-induced depressive behaviour, as measured with the forced swim test, was prevented by phagocytic microglia depletion. These data show that microglia are important effector cells that underlie the inflammatory changes associated with chronic behavioural alterations such as those associated with depression in humans.

## Introduction

Despite intense research, microglia remain relatively ‘mysterious’ cells, and only recent results have begun to provide answers to basic questions such as their developmental origin (Ginhoux et al. 2010; Kierdorf et al. 2013). However, one long standing and established aspect of microglia biology is their involvement in fighting brain inflammation and injury and more recently in the triggering of numerous brain disorders [see (Salter and Stevens 2017) for review]. Research has linked neuroinflammation as a major contributing factor to a number of neurodegenerative diseases and mood disorders such as depression [see (Hurley and Tizabi 2013) for review]. Experimental studies using systemic infection to induce a neuroinflammatory response in rodents has used the bacterial cell wall product lipopolysaccharide (LPS), a potent activator of inflammatory mediators both in the periphery and the brain. A single injection of this immunogen has been shown to reliably induce depressive-like behaviour in rodents (Bay-Richter et al. 2011; Frenois et al. 2007; O’Connor, Lawson, et al. 2009; Sekio and Seki 2015) and we have recently confirmed that this could be related to microglial activation (Aguilar-Valles et al. 2014).

Microglial activation is the principal component of neuroinflammation in the central nervous system (CNS), providing the first line of defence whenever brain injury or disease occurs (Block and Hong 2005; Glass et al. 2010). Increasing evidence points to a heterogeneous status of microglial activation in the CNS. Indeed, some authors categorize microglia into two opposite activation states, M1 and M2 phenotypes, which can produce either cytotoxic or neuroprotective effects (Tang and Le 2016). LPS is known as a representative M1 polarization inducer (Chhor et al. 2013) and M1 microglia are associated with the production of pro-inflammatory cytokines such as interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF) $\alpha$  (Antonietta Ajmone-Cat et al. 2013). This initial pro-inflammatory response is not detrimental to the brain, indeed it is a necessary step in wound repair promoting the killing of invading organism and removing debris to “clean” the damaged area (Soehnlein and Lindbom 2010). This response is then shifted to an anti-inflammatory state, driven by M2-polarized microglia and the regulation of various anti-inflammatory factors in order to antagonize the M1 pro-inflammatory response. This M2 response promotes a number of mechanistic steps including phagocytosis of cell debris and misfolded proteins, extracellular

matrix reconstruction, tissue repair and angiogenesis (Varin and Gordon 2009). Thus, when there is a proper transition from the M1 to M2 phenotype, the damage can be efficiently repaired [see (Nakagawa and Chiba 2014; Tang and Le 2016) for review].

In addition to inducing cytokines in the brain, LPS has also been shown to activate the Tryptophan-pathway, associated with the development of depressive-like behaviour in rodents (O'Connor, Lawson, et al. 2009). Specifically, LPS was demonstrated to be a potent activator of indoleamine 2,3-dioxygenase (IDO), the metabolic rate limiting enzyme of the tryptophan pathway (Corona et al. 2013; Wang et al. 2010). The principle role of this enzyme is the conversion of tryptophan, the precursor of serotonin (5-HT) to kynurenin, the metabolites of which have toxic effects on brain function [see (Capuron and Miller 2004; Schmidt and Schultze 2014) for review]. As such, IDO expression in the brain is now been universally used as a biomarker of inflammation-induced depression-like behaviour in rodents and following drug therapy with interferon(IFN)- $\alpha$  in humans (O'Connor, Lawson, et al. 2009; Raison et al. 2010). Interestingly, IDO has been shown to be induced in microglia by IFN $\gamma$  -producing T helper (Th) 1 cells, initiating a negative feedback loop to reduce neuroinflammation (Kwidzinski and Bechmann 2007; Yadav et al. 2007).

In humans, depressive symptoms often follow various bacterial and viral infections (Yirmiya et al. 1999), which are also associated with activation of microglial cells (Rock et al. 2004). Experiments in animal models provided more direct evidence for a role of microglia in depression (Corona et al. 2013; Fenn et al. 2014; Henry et al. 2008; O'Connor, Andre, et al. 2009; O'Connor, Lawson, et al. 2009). In the current study, we investigated the specific contribution of microglial cells to the overall CNS inflammatory response in mice treated with an injection of LPS. For this, we depleted phagocytic microglia using mannoseylated liposome-mediated intracellular delivery of dichloromethylene-bisphosphonate (Clodronate) injected in the hippocampal CA3 region prior to LPS treatment. CA3 region is associated with cognitive and behaviour alteration in depression as well as susceptibility to changes in inflammatory milieu [see (Campbell and Macqueen 2004) for review]. As expected, LPS induced a significant increase in microglia activation abolished by the Clodronate treatment. The analysis of inflammatory mediators' expression revealed a significant increase in the pro-inflammatory cytokines IL-1 $\beta$  and TNF $\alpha$  and the anti-inflammatory cytokine IL-10 after

LPS treatment, all of which were attenuated by liposome-mediated phagocytic microglia depletion except for IL-1 $\beta$ , the expression of which was potentiated. In addition, LPS induced an increase of IDO expression and despair-like behaviour, which were respectively completely prevented and attenuated by the intracerebral administration of mannosylated clodronate liposomes (MCLs). Interestingly, LPS-induced behavioural alterations, as measured by increased immobility time in the forced swim test (FST), highly correlated to IDO expression levels. Altogether, this data suggests that phagocytic microglia may be important effectors underlying the inflammatory changes associated with depressive-like behaviours.

## Materials and Methods

### *Animals*

All protocols were approved by McGill University's Animal Care Committee under the guidelines of the Canadian Council on Animal Care. Male C57/BL6 mice, 9-10 weeks old (Charles River, Montreal, QC), were housed individually for ten days prior to the start of the experiments, to habituate to new housing conditions, under a 12hrs light cycle (8am-8pm) with *ad libitum* access to food and water. Body weight and food intake were measured daily 24 hours, 48 hours before Saline/LPS treatment and 96 hours after liposome intervention and before the FST.

### *Intracerebral liposome administration*

Following anesthesia with 3-4% isoflurane in 70:30% N<sub>2</sub>O:O<sub>2</sub>, mice were placed in a stereotaxic frame and anesthesia was maintained with 2% isoflurane. In order to deplete phagocytic microglial cells, mannosylated clodronate (10 mg/ml) liposomes (MCLs) (or mannosylated PBS liposomes as control) (Encapsula Nanosciences, USA) were intracerebrally administered (Miron et al. 2013) (Control: n=16; MCLs: n=14 Saline/LPS). Liposomes were gently rocked to yield a homogeneous suspension before intracerebral injection. Using a finely drawn glass micro capillary, 50 µm diameter tip, control liposome or MCLs (0.5µL per side) were stereotactically injected into the CA3, through a burr hole in the skull (coordinates: Anterior-Posterior: -3.08 mm, Medial-Lateral: +/- 3.25 mm, Dorsal-Ventral: -3.75 mm). Following injection, the scalp incision was sutured and animals allowed to recover from anesthesia.

### *Immune activation*

48 hours after liposome intervention, mice from Control and MCLs groups were treated with a single intraperitoneal (i.p.) injection of LPS (2.5 mg/kg Escherichia coli 0111:B4; cat: L-2630; Sigma-Aldrich, Oakville, ON, Canada) or pyrogen-free saline (IP, 4 mL/kg) (Saline: n=8 Control, n=7 MCLs; LPS: : n=7 n=8 Control, n=7 MCLs). The dose of LPS used is one that we have previously confirmed to induce despair-like behaviour in mice (Aguilar-Valles et al. 2014).

***Forced swim test***

48 hours after Saline or LPS treatment (96 hours after liposome intervention), mice were placed individually into glass cylinders (height:50 cm, diameter: 10 cm) containing water up to a 40 cm mark (at 23-25 °C) for 6 min, using our previously described methods (Aguilar-Valles et al. 2014). A mouse was judged to be immobile when it floated in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the 6 min of the testing period.

***RNA extraction and gene expression Reverse Transcription-PCR***

After behavioural testing, animals were killed by terminal anesthesia [rodent cocktail (Ketamine 100mg/mL, Xylazine 20mg/mL and Acepromazine 10mg/mL; 0.1mL/100g)]. The brains were quickly removed and hippocampi were dissected, frozen on powdered dry ice and kept at -80 °C until analysis. Tissues were homogenized in TRIzol (Invitrogen) for RNA extraction according to the manufacturer's instructions. RT reaction was performed as previously described (Maric, Woodside, and Luheshi 2014). Briefly, 1 µg of total RNA was transcribed by heating in a Gene Amp PCR System 9700 Thermocycler (Applied Biosystems) with 5 µL DEPC water, 1 µL random hexamers (Applied Bioscience), and 1 µL dNTPs (Sigma-Aldrich) at 65 °C for 10 min. cDNA was synthesized by incubating with primer mix [with 2 µL 0.1 dithiothreitol (Invitrogen), 1 µL murine myeloleukemia virus reverse transcriptase (Invitrogen), 4 µL first-strand buffer (Invitrogen) and 2 µL distilled water)] in the thermocycler at 37 °C for 1 h and then at 90 °C for 5 min in a total volume of 20 µL. All RNA samples were reverse transcribed simultaneously to minimize inter-assay variation. Following reverse transcription, the product was stored at -20 °C. Real-time RT-PCR was performed using Taqman gene expression assays for IDO (cat #Mm00492586\_m1), Arg1 (cat #Mm00475988\_m1), Ptgs2 (cat #Mm00478374\_m1), IL-10 (cat #Mm00439614\_m1), IL-1β (cat #Mm00446190\_m1), TNFα (cat #Mm00443258\_m1) and 18S (cat #4352930E) as the endogenous control, purchased from Applied Biosystems. In a total volume of 20 µL, each sample-contained 2 µL of prepared cDNA described above, 1 µL primer/probe mixture (Applied Biosystems), and 10 µL taqMan PCR master mix (Applied Biosystems). cDNA was amplified in duplicates by quantitative real-time PCR

(7500 Real-Time PCR System, Applied Biosystems). Relative quantitative measurement of target gene levels was performed using the  $\Delta\Delta C_t$  method, where  $C_t$  is the threshold concentration. Differences in expression were calculated using the formula fold change =  $2^{(-\Delta\Delta C_t)}$  and expressed as a fold increase relative to the control condition.

### ***Statistical Analysis***

Two-way repeated measures Analysis of Variance (ANOVA) with Tuckey post-hoc tests were used to assess the effect of liposome intervention (Control and MCLs) and treatment (Saline and LPS) and their potential interaction on body weight loss and food intake across time using SPSS Software. Two-way ANOVA with Tuckey post-hoc tests were used to assess the effect of liposome intervention and treatment and their potential interaction on behaviour and genes expression in Graphpad Prism 6 Software. Pearson correlation coefficient analysis was used to analyze the correlations between the relative expression of IDO and the behavioural measurements on IBM SPSS Statistics software. All data are presented as the mean value for each treatment group  $\pm$  SEM.

## Results

### ***LPS treatment but not liposome-mediated phagocytic microglia depletion affects body weight and food intake.***

Systemic LPS induces an array of ‘sickness-type’ responses including loss of appetite and body weight, commonly used parameters for assessing infection (Privitera, Misenheimer, and Doraiswamy 2013). In the current study, and as expected, body weight loss and daily food intake were significantly affected by LPS treatment (Body weight loss:  $F_{(1, 26)} = 70.010$ ,  $p < 0.001$ ; [Figure II.1a](#)) (Food intake:  $F_{(1, 26)} = 203.487$ ,  $p < 0.001$ ; [Figure II.1b](#)). LPS-treated animals had an overall increased body weight loss (1.913 +/-0.160 g vs. 0.575 +/-0.160 g) and decreased food intake (3.111 +/-0.062 g vs. 4.369 +/-0.062 g) when compared to Saline-treated animals. Moreover, there was a significant interaction between time post intervention and treatment (Body weight loss:  $F_{(3, 78)} = 92.547$ ,  $p < 0.001$ ; [Figure II.1a](#)) (Food intake:  $F_{(3, 78)} = 62.053$ ,  $p < 0.001$ ; [Figure II.1b](#)), with LPS-treated animals’ body weight and food intake being significantly different from Saline-treated animals only 72 and 96 hours post intervention (corresponding to post LPS treatment timepoints). These results verified the effectiveness of this dose of LPS as an activator of the immune system which is universally known to mediate the sickness type responses including those measured here (Dantzer et al. 2008). Unlike the LPS, intracerebral administration of mannosylated vehicle liposomes (Control) or clodronate liposomes had no significant effect on body weight loss ( $F_{(1, 26)} = 0.677$ ,  $p > 0.05$ ; [Figure II.1a](#)) and food intake ( $F_{(1, 26)} = 0.408$ ,  $p > 0.05$ ; [Figure II.1b](#)).

### ***Liposome-mediated phagocytic microglia depletion prevents LPS-induced immobility in the forced swim test and attenuates LPS-induced IDO mRNA levels.***

To test the effects of liposome intervention and LPS treatment on despair-like behaviour and mRNA levels of *IDO* [an enzyme which plays a major role in the pathophysiology of inflammation-induced depressive symptoms (Wichers et al. 2005)], we performed a FST 96 hours after vehicle (Control) or clodronate liposomes (MCLs) administration, and 48 hours after Saline or LPS treatment. Following this behavioural test, the animals were immediately sacrificed, brains extracted and *IDO* mRNA expression measured in the hippocampi (structure of liposome administration). Liposome intervention did not have a significant main

effect on immobility time during the FST session ( $F_{(1, 26)} = 0.737, p > 0.05$ ; [Figure II.2a](#)), but LPS treatment did ( $F_{(1, 26)} = 17.17, p < 0.001$ ; [Figure II.2a](#)), and there was a significant interaction between liposome intervention and LPS treatment ( $F_{(1, 26)} = 6.911, p = 0.014$ ; [Figure II.2a](#)). *Post-hoc* tests showed that LPS treatment increased the percentage of immobility time in Control animals while this effect was prevented in MCLs animals (Control-Saline vs. Control-LPS:  $p = 0.002$ ; MCLs-Saline vs. MCLs-LPS:  $p > 0.05$ ). mRNA levels of *IDO* were affected by both liposome intervention ( $F_{(1, 26)} = 633.7, p < 0.001$ ; [Figure II.2b](#)) and LPS treatment ( $F_{(1, 26)} = 10618, p < 0.001$ ; [Figure II.2b](#)) and there was a significant interaction between liposome intervention and treatment ( $F_{(1, 26)} = 641.9, p < 0.001$ ; [Figure II.2b](#)). LPS induced a significant increase in the mRNA expression of *IDO* when compared to Saline-treated animals, regardless of liposome intervention (Control/MCLs-Saline vs. Control/MCLs-LPS:  $p < 0.001$ ). However, this effect of LPS was significantly attenuated after administration of MCLs when compared to control animals having received an administration of vehicle liposomes in hippocampal CA3 region (Control-LPS vs. MCLs-LPS:  $p < 0.001$ ). Relative levels of *IDO* mRNA and immobility time in the FST were strongly positively correlated in LPS treated animals (Control-LPS:  $r = 0.903; p = 0.002$ ; MCLs-LPS:  $r = 0.919; p = 0.003$ ; [Figure II.2c](#)).

***Liposome-mediated phagocytic microglia depletion respectively attenuates and prevents LPS-induced Iba1 and Arg1 mRNA levels but did not affect LPS-induced Ptgs2 mRNA levels.***

To investigate the effects of liposome intervention and LPS treatment on microglia, we measured the mRNA expression of several microglial markers. mRNA levels of *Iba1* (commonly used as a marker for microglial activation) and *Arg1* (marker of M2 microglial polarization) were affected by both liposome intervention (*Iba1*:  $F_{(1, 26)} = 4.621, p = 0.041$ ; [Figure II.3a](#)) (*Arg1*:  $F_{(1, 26)} = 5.480, p = 0.027$ ; [Figure II.3b](#)) and LPS treatment (*Iba1*:  $F_{(1, 26)} = 4.508, p = 0.043$ ; [Figure II.3a](#)) (*Arg1*:  $F_{(1, 26)} = 11.45, p = 0.002$ ; [Figure II.3b](#)). LPS treatment increased the mRNA levels of *Iba1* and *Arg1* in vehicle liposomes administered animals (Control), and this effect was completely prevented in animals that received an intracerebral administration of MCLs (*Iba1*: Control-Saline vs. Control-LPS:  $p = 0.019$ ; MCLs-Saline vs.

MCLs-LPS:  $p>0.05$ ) (Arg1: Control-Saline vs. Control-LPS:  $p=0.006$ ; MCLs-Saline vs. MCLs-LPS:  $p>0.05$ ). Moreover, there was an interaction between liposome intervention and LPS treatment for mRNA levels of *Iba1* ( $F_{(1, 26)} = 5.430$ ,  $p=0.027$ ; [Figure II.3a](#)). *Iba1* mRNA expression was significantly lower in LPS-treated animals following MCLs administration when compared to LPS-treated control animals (Control-LPS vs. MCLs-LPS:  $p<0.001$ ). Liposome intervention did not have a significant main effect on mRNA levels of *Ptgs2* (marker of M1 microglial polarization) ( $F_{(1, 26)} = 0.019$ ,  $p>0.05$ ; [Figure II.3c](#)), but LPS treatment did ( $F_{(1, 26)} = 51.96$ ,  $p<0.001$ ; [Figure II.3c](#)). LPS treatment induced increased levels of *Ptgs2* mRNA regardless of liposome intervention (Control/MCLs-Saline vs. Control/MCLs-LPS:  $p<0.01$ ).

***Liposome-mediated phagocytic microglia depletion potentiates LPS-induced IL-1 $\beta$  mRNA levels and attenuates LPS-induced TNF $\alpha$  and IL-10 mRNA levels.***

Given that microglia represent a significant source and target of cytokines, we assessed the effects of liposome intervention and LPS treatment on pro-inflammatory cytokines IL-1 $\beta$ , TNF $\alpha$  mediators of sickness and depressive-like behaviours (Dantzer and Kelley 2007; Hauser et al. 2002; Raison and Miller 2013) as well as the anti-inflammatory cytokine IL-10. mRNA levels of *IL-1 $\beta$* , *TNF $\alpha$*  and *IL-10* were significantly affected by both, liposome administration (IL-1 $\beta$ :  $F_{(1, 26)} = 134.4$ ,  $p<0.001$ ; [Figure II.4a](#)) (TNF $\alpha$ :  $F_{(1, 26)} = 15.04$ ,  $p<0.001$ ; [Figure II.4b](#)) (IL-10  $F_{(1, 26)} = 27.53$ ,  $p<0.001$ ; [Figure II.4c](#)) and treatment (IL-1 $\beta$ :  $F_{(1, 26)} = 708.3$ ,  $p<0.001$ ; [Figure II.4a](#)) (TNF $\alpha$ :  $F_{(1, 26)} = 423.2$ ,  $p<0.001$ ; [Figure II.4b](#)) (IL-10  $F_{(1, 26)} = 216.3$ ,  $p<0.001$ ; [Figure II.4c](#)). Moreover, there was a significant interaction between liposome administration and treatment for these three cytokines mRNA expression (IL-1 $\beta$ :  $F_{(1, 26)} = 86.32$ ,  $p<0.001$ ; [Figure II.4a](#)) (TNF $\alpha$ :  $F_{(1, 26)} = 43.91$ ,  $p<0.001$ ; [Figure II.4b](#)) (IL-10  $F_{(1, 26)} = 25.99$ ,  $p<0.001$  [Figure II.4c](#)). *Post-hoc* analyses showed that LPS treatment induced increased mRNA levels of IL-1 $\beta$ , TNF $\alpha$  and IL-10 when compared to Saline treatment (Control/MCLs-Saline vs. Control/MCLs-LPS:  $p<0.001$ ). While intracerebral administration of MCLs potentiated the effect of LPS on IL-1 $\beta$  mRNA, it attenuated the effect of LPS on TNF $\alpha$  and IL-10 mRNA levels (Control-LPS vs. MCLs-LPS:  $p<0.001$ ).

## Discussion

In the present study, we demonstrate that an administration of a single systemic dose of LPS induces despair-like behaviour as well as classic sickness-type responses such as reduction in food intake and body weight. At the molecular level in the brain, LPS significantly upregulated the expression of IDO, a rate limiting enzyme associated with the tryptophan system. The expression of IDO correlated significantly with immobility time taken in the FST, used for assessing depression-like behaviour in rodents. Most importantly, we demonstrated that liposome-mediated phagocytic microglia depletion attenuated these LPS-induced changes indicating direct involvement of microglia. Finally, we assessed the effect of LPS treatment and liposome intervention on different molecular signals associated with microglial function including M1 and M2 microglia markers, pro-and anti-inflammatory cytokines and showed that while phagocytic microglia depletion potentiated the LPS-induced *IL-1 $\beta$*  mRNA expression, it attenuated those of TNF $\alpha$  and IL-10. These observations indicate a strong association between phagocytic microglia, inflammatory mediators and development of despair-like behaviour following LPS treatment.

Microglia mediate multiple facets of neuroinflammation, including cytotoxicity, repair, regeneration, and immunosuppression due to their ability to acquire diverse activation states, or phenotypes. LPS is a polarization inducer of the M1 microglial phenotype. Indeed, as already shown *in vitro*, LPS increases gene expression of known cytotoxic M1 markers including *Ptgs2* and causes a decrease in the expression of the M2 markers *Arg1* hours after treatment (Chhor et al. 2013). In our study, i.p. administration of LPS resulted in microglial activation, as measured by increased expression of *Iba1* marker. Interestingly, while LPS treatment induced increased levels of *Ptgs2*, it also induced increased levels of *Arg1*. Environment plays a crucial role in controlling the shift from classical to alternative activation (Chhor et al. 2013). With LPS, the domination of M1 microglia is mainly due to the high levels of pro-inflammatory cytokines present (Cazareth et al. 2014; Erickson and Banks 2011; Qin et al. 2008). Importantly, polarized microglia are not locked in a particular state; microglia are plastic cell types that can be altered if their environment changes (Davis et al. 2013). An example of this is changes in the levels of 2-Arachidonoyl-glycerol (2-AG).

This is an endogenous cannabinoid biosynthesized in various types of cells including microglia, and binds to two distinct cannabinoid receptors (CB<sub>1</sub> and CB<sub>2</sub>) (Freund, Katano, and Piomelli 2003; Varga et al. 2018). CB<sub>1</sub> receptors are constitutively expressed in various tissues and higher expressions are found in neural cells while CB<sub>2</sub> receptors are most specifically expressed in glia (Stella 2010) and this expression significantly increases during microglial activation (Maresz et al. 2005). Agonistic stimulation of CB<sub>1</sub> receptor by 2-AG can induce internalization and down-regulation of CB<sub>1</sub> (Atwood et al. 2012). Conversely, a CB<sub>2</sub> receptor agonist, reduced glial activation and inhibited pro-inflammatory cytokines (Javed et al. 2016). Taken together, it appears that 2-AG induces down-regulation of CB<sub>1</sub> and up-regulation of CB<sub>2</sub> conversely. Thus, following LPS challenge, 2-AG released from M1 microglia might induce down-regulation of CB<sub>1</sub> and stimulate M2 polarization of microglia *via* CB<sub>2</sub>, explaining the increased expression levels of the M2 microglia marker measured 48 hours after LPS injection in our study. An additional possibility is one that involves neutrophils. Indeed, these cells could also play a role in the M2 polarization measured 48 hours after LPS treatment. In a previous study we have demonstrated that a single systemic injection of LPS (as used in the current study) triggers a significant translocation of neutrophils from the peripheral circulation into the brain (Aguilar-Valles et al. 2014). We showed that brain neutrophil numbers increase 24, 48 and 72 hours after the i.p. LPS treatment reaching a peak at the 24 hours time point, and decreasing by the 48h (Aguilar-Valles et al. 2014). Systemic inflammation involves the rapid recruitment of neutrophils to the site of damage to promote recruitment of inflammatory monocytes and to potentiate the pro-inflammatory environment allowing for an effective resolution of inflammation (Mantovani, Bonecchi, and Locati 2006). Under normal conditions, neutrophils undergo apoptosis after performing this action (Fox et al. 2010) and are thereafter ingested by macrophages. Clearance of apoptotic neutrophils prompts a switch from a pro- to an anti-inflammatory macrophage phenotype (Fadok et al. 1998; Michlewska et al. 2009). It is tempting to suggest that a similar mechanism exists in the brain involving microglial switch in the same direction, however, to the best of our knowledge, evidence to support this notion is not available.

One of the most interesting results from the present study relate to the induction of IDO in the brain. Previous studies have demonstrated that animals subjected to immune challenge with LPS, a well-established animal model of inflammation-induced depression (Aguilar-Valles et al. 2014; Frenois et al. 2007; Ge et al. 2015; O'Connor, Lawson, et al. 2009; Sulakhiya et al. 2016), present increases in IDO expression in brain areas related to mood regulation, such as the hippocampus, hypothalamus and amygdala (O'Connor, Andre, et al. 2009; O'Connor, Lawson, et al. 2009). In this study, we showed strong correlation between IDO expression levels in the hippocampus and depressive-like behaviour, both induced by LPS treatment and respectively attenuated and prevented by administration of MCLs. Whether the induction of this enzyme is in any way related with cytokine production in the brain remains to be determined, however O'Connor et al showed that the anti-inflammatory tetracycline derivative minocycline, that attenuates LPS-induced expression of pro-inflammatory cytokines results in a blockade of IDO activation (O'Connor, Lawson, et al. 2009).

We also evaluated the effects of LPS treatment and liposome intervention on brain pro-inflammatory cytokines IL-1 $\beta$ , TNF $\alpha$  and the anti-inflammatory cytokine IL-10. As expected, increased expression levels of IL-1 $\beta$  and TNF $\alpha$  were measured following LPS treatment (Cazareth et al. 2014; Erickson and Banks 2011; Qin et al. 2008). The M1 activation stage has been associated with the production of pro-inflammatory cytokines (Colton 2009; Colton and Wilcock 2010) and our results are in perfect accordance with the increased expression of the M1 microglia marker *Ptgs2* measured in LPS-treated animals when compared to saline controls. We also observed increased expression levels of the anti-inflammatory cytokine IL-10 that might be explained by increased polarization towards the M2 microglia as indicated by increased expression of *Arg1* in microglia (Colton 2009; Colton and Wilcock 2010).

Clodronate is a bisphosphonate drug used to treat osteolytic disease, which can cause irreversible metabolic damage and apoptosis at high intracellular concentrations (Rooijen and Sanders 1994). In this study, we depleted M2 cells using mannosylated clodronate liposomes (MCLs) that bind the mannose receptor which is upregulated following M2 polarization (Bedi et al. 2013; Cherry, Olschowka, and O'Banion 2014) and are

phagocytosed by M2 phagocytic microglia, thereby inducing apoptosis within one day *via* clodronate-mediated depletion of intracellular iron (Miron et al. 2013; Torres et al. 2016). Selective and localized M2 microglia depletion after intracerebral administration of MCLs was confirmed by others (Miron et al. 2013; Torres et al. 2016), as well as our results, as shown by the specific effect of MCLs on *Arg1* expression levels while it had no effect on *Ptgs2* expression. As expected, liposome-mediated phagocytic microglia depletion attenuated LPS-induced IL-10 expression. Interestingly, while M1 microglia were not affected by MCLs administration, this treatment caused an exacerbation of LPS-induced expression levels of pro-inflammatory cytokine IL-1 $\beta$ , supporting previous findings of reciprocal regulation between M1 and M2 polarization and associated cytokines (Das et al. 2001; Jang et al. 2013; Miron et al. 2013). As already shown in primary mixed glial cell cultures, IL-10 inhibits LPS-induced IL-1 $\beta$  (Sawada et al. 1999). M2 microglial depletion resulting in decreased levels of anti-inflammatory cytokines will reduce/remove the influence of this mediator subsequently resulting in a more pro-inflammatory environment, exemplified by the potentiation of the LPS-induced IL-1 $\beta$  levels.

Whilst this can explain the relative association between IL-1 $\beta$  and IL-10, the reduction in TNF $\alpha$  in our experiment is rather puzzling. It is largely acknowledged that TNF $\alpha$  is defined as a pro-inflammatory cytokine and whilst some have argued that it may also exert some anti-inflammatory activity (Long et al. 1990) this view was never largely accepted (Luheshi et al. 1997). Whilst our results suggest that in this case, TNF $\alpha$  may be acting as an anti-inflammatory cytokine this needs to be confirmed. This view would actually correspond with the role described for TNF $\alpha$  in depression. This cytokine plays an important role in modulating mood behaviour (Miller, Maletic, and Raison 2009; Dowlati et al., 2010; Howren et al., 2009). Various studies have suggested that TNF $\alpha$  induces neurochemical processes that are reminiscent of depression which is associated with deficiency in serotonergic transmission. TNF $\alpha$  reduces serotonin levels by lowering the availability of its precursor tryptophan and induces the activity of IDO, an enzyme that degrades tryptophan (Lestage et al. 2002) which we have shown here to be upregulated by LPS and inhibited by MCL treatment. TNF $\alpha$  also increases the reuptake of serotonin by enhancing the activity of serotonin transporters (Zhu, Blakely, and Hewlett 2006). In addition to its effects on

monoamine system, TNF $\alpha$  also regulates the neuro-endocrine system (Ma, Zhang, and Baloch 2016) and has been shown to stimulate the hypothalamic pituitary adrenal (HPA) axis (Dunn 2000). Altogether, it suggests that in our model of LPS treatment, prevention of depressive-like behaviour *via* phagocytic microglia depletion are likely through attenuation of TNF $\alpha$  expression levels that would then act on monoamine neurotransmitters and/or HPA axis.

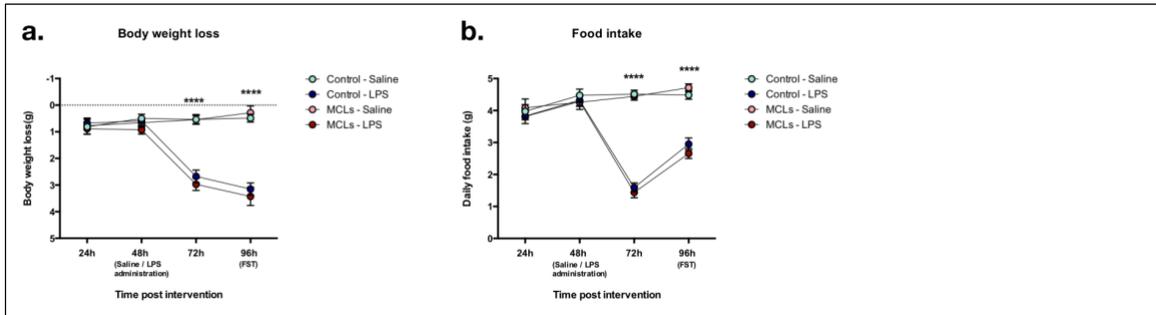
In summary, our studies provide strong evidence implicating a role for microglia in mediating LPS-induced depressive-like behaviour and identified phagocytic microglia depletion as a way to prevent such behavioural alterations. This lends support to the debatable notion that M2 microglia are neuroprotective (Cherry, Olschowka, and O'Banion 2014).

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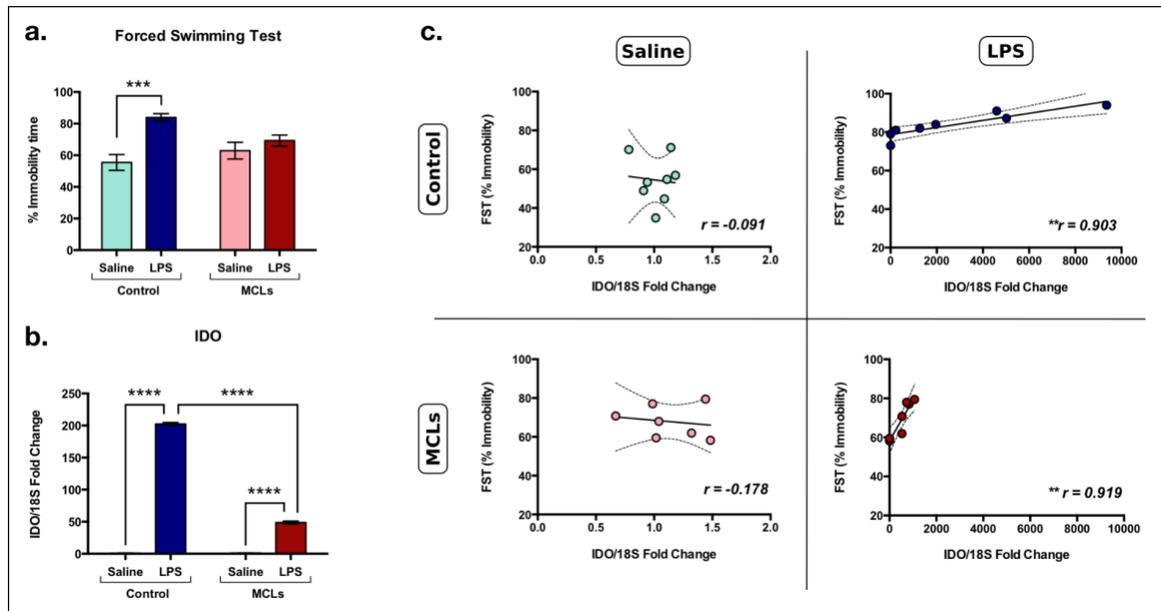
## Figures

**Figure II.1. Effect of liposome-mediated phagocytic microglia depletion and LPS administration on body weight loss and food intake.**



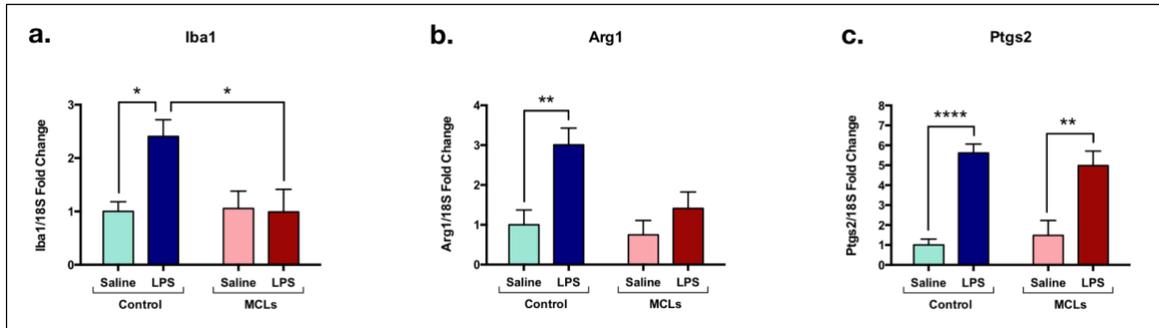
**Figure II.1.** Saline or LPS was injected *i.p.* 48 hours after intracerebral administration of control liposomes (Control) or mannosylated clodronate liposomes (MCLs), and changes in body weight (a) and food intake (b) were measured at baseline then, 24, 48, 72 and 96 hours after liposome intervention. LPS treatment but not liposome intervention resulted in decreased body weight and daily food intake. (Control:  $n=8$  Saline/LPS; MCLs:  $n=7$  Saline/LPS). Data displayed represent the mean value  $\pm$  SEM (\* $P<.05$ , \*\* $P<.01$ , \*\*\* $P<.001$ , \*\*\*\* $P<.0001$ ).

**Figure II.2. Liposome-mediated phagocytic microglia depletion prevents LPS-induced immobility in the forced swim test and attenuates LPS-induced IDO synthesis.**



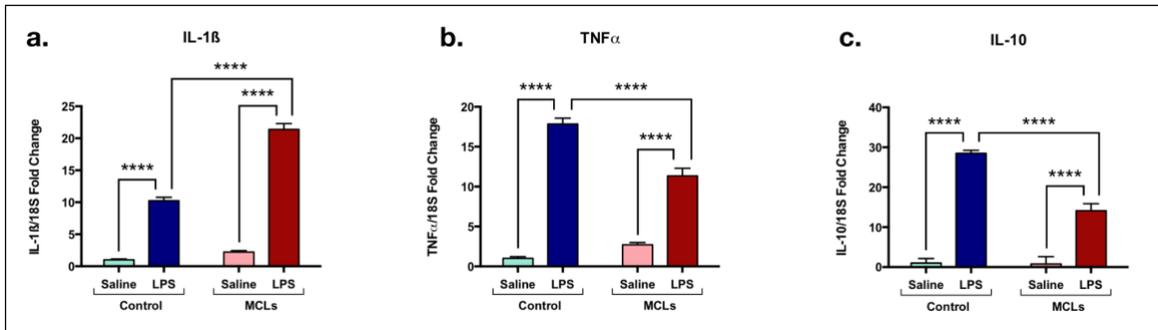
**Figure II.2.** Immobility time in the forced swim test (a) and mRNA levels of IDO (b) were measured 48 hours after Saline or LPS treatment on animals co-treated with intracerebral administration of vehicle liposomes (Control) or mannosylated clodronate liposomes (MCLs). LPS treatment induced an increased percentage of time spent in immobility in the forced swim test which was prevented by MCLs (a). IDO mRNA levels in the hippocampus were significantly induced by LPS treatment and this treatment effect was attenuated by MCLs intervention (b). Expression levels of IDO mRNA relative to the 18S endogenous control gene were correlated to immobility time in the FST (c). Positive correlations were observed in LPS-treated animals. (Control:  $n=8$  Saline/LPS; MCLs:  $n=7$  Saline/LPS). Data displayed represent the mean value  $\pm$  SEM (\*\* $P < .001$ , \*\*\*\* $P < .0001$ ).

**Figure II.3. Liposome-mediated phagocytic microglia depletion prevents LPS-induced increase of *Iba1* and *Arg1* mRNA levels but did not affect LPS-induced increase of *Ptgs2* mRNA levels.**



**Figure II.3.** mRNA levels of microglial markers were measured 48 hours after Saline or LPS treatment on animals co-treated with an intracerebral administration of vehicle liposomes (Control) or mannosylated clodronate liposomes (MCLs). LPS treatment induced a significant increase in *Iba1* (a), *Arg1* (b) and *Ptgs2* (c) mRNA levels. Intracerebral administration of MCLs prevented the effect of LPS on *Iba1* and *Arg1* mRNA levels whilst having no effect on the LPS-induced *Ptgs2* mRNA levels. (Control:  $n=8$  Saline/LPS; MCLs:  $n=7$  Saline/LPS). Data displayed represent the mean value  $\pm$  SEM (\* $P<.05$ , \*\* $P<.01$ , \*\*\* $P<.001$ , \*\*\*\* $P<.0001$ ).

**Figure II.4. Liposome-mediated phagocytic microglia depletion potentiates LPS-induced increase of *IL-1 $\beta$*  mRNA levels and attenuates LPS-induced increase of *IL-10* and *TNF $\alpha$*  mRNA levels.**



**Figure II.4.** mRNA levels of *IL-1 $\beta$* , *TNF $\alpha$*  and *IL-10*, cytokines were measured 48 hours after Saline or LPS treatment on animals co-treated with an intracerebral administration of control liposomes or mannosylated clodronate liposomes (MCLs). LPS treatment induced a significant increase of *IL-1 $\beta$*  (a), *TNF $\alpha$*  (b) and *IL-10* (c) mRNA levels. Intracerebral administration of MCLs attenuated the effect of LPS on *TNF $\alpha$*  (b) and *IL-10* (c) mRNA levels while it potentiated LPS-induced *IL-1 $\beta$*  mRNA levels (a). (Control: n=8 Saline/LPS; MCLs: n=7 Saline/LPS). Data displayed represent the mean value  $\pm$  SEM (\*\*\*\*P<.0001).

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### **Chapter III:**

**Early maternal immune activation alters microglia developmental trajectory and induces long-term behavioural alterations in the offspring in a sexually dimorphic manner**

### **III.1. Preface**

The work presented in the last chapter (Chapter 2) reflected a strategy that was designed to address the question of how microglia contributed to a short-term single systemic challenge with a bacterial immunogenic stimulus, LPS. Of particular interest was the polarization effect that the LPS had on the microglial population and how that contributed to the mounting and ultimate resolution of the inflammatory response. In those studies, the changes in microglial phenotypes were assessed in conjunction with despair-like behaviour induced by the LPS. Most importantly we linked the microglial activity with a biochemical marker, IDO, used experimentally to assess the development of depression-like behaviour confirming a potential role for these cells in the development of the behavioural changes observed.

Building on these findings and given the recently described role of microglia in neurodevelopment, we investigated the sustained effect of maternal immune activation on microglia population and behaviour of the offspring. For this, we utilized a maternal immune activation (MIA) model using the same LPS used in Chapter 2 administered to mice at a relatively early stage of gestation when microglia start colonizing the fetal brain.

## **III.2. Manuscript**

**Title: Early maternal immune activation alters microglia developmental trajectory and induces long-term behavioural alterations in the offspring in a sexually dimorphic manner**

**Chloé Lacabanne<sup>1,2</sup>, Anouk Benmamar--Badel<sup>1,2</sup>, Quentin Leyrolle<sup>2</sup>, Maude Bordeleau<sup>1</sup>, Sophie Layé<sup>2\*</sup> and Giamal N. Luheshi<sup>1\*</sup>**

<sup>1</sup> Douglas Hospital Research Centre, Department of Psychiatry, McGill University, Quebec, Canada H4H 1R3

<sup>2</sup> Nutrition et Neurobiologie Intégrée, UMR 1286, INRA, Université de Bordeaux, Bordeaux, France

\*Equal contribution

## Abstract

Recent discoveries have profoundly changed the conventional view of microglia being mere passive immune sentinels to critical and active sculptors of neuronal networks through the removal of superfluous synapses. The disruption of this process (known as “pruning”), following exposure to environmental risk factors such as infection or inflammation, had been linked to neurodevelopmental defects resulting in aberrant behaviours associated with mental disorders in humans. Whilst the majority of the microglial pruning activity has been reported to occur early in the postnatal period, microglia are also involved in mediating neuronal proliferation and differentiation, as well as in the control of neuronal death that occurs as part of the neurodevelopmental process. Moreover, recent reports including some from our laboratory suggested that targeting microglia during the embryonic period can have serious long-term consequences in the behaviour of the offspring. In the current study, we utilized a maternal immune activation (MIA) model using a single injection of Lipopolysaccharide (LPS) in mice administered at gestational day (GD)9.5 the time point when microglia colonize the fetal brain. Using FACS analysis of fetal brains collected at GD14.5 and GD18.5 (representing different developmental time points in the microglial ontogeny) we detected LPS-induced changes in microglial maturation. Postnatally, the offspring of the LPS-treated mothers exhibited significantly altered social/communicative behaviour. In adulthood, LPS-treated offspring displayed increased repetitive behaviour and altered social preference and social memory. Interestingly, these behavioural results were different in males compared to female progeny suggesting that sexual dimorphism is a feature of the changes triggered by the LPS *in utero*. These results further confirm our earlier findings and show that exposure to immunogenic stimuli early in gestation disrupts normal microglial development possibly resulting in disrupted network formation in the fetal brain and subsequent behavioural anomalies mostly in the male offspring.

## Introduction

Recent advances in microglial biology, demonstrated a new role for these cells in early brain development. Specifically, microglia were shown to play a crucial physiological function in the building and wiring of the developing central nervous system (CNS): they are involved in mediating neuronal proliferation and differentiation, as well as in the control of neuronal death that occurs as part of the neurodevelopmental process (Antony et al. 2011; Cunningham, Martinez-Cerdeno, and Noctor 2013; Dalmau et al. 2003; Mazaheri et al. 2014; Wakselman et al. 2008), formation of synapses (Kim et al. 2017; Paolicelli et al. 2011; Schafer et al. 2012) and establishment of neuronal circuits during early development (Hoshiko et al. 2012; Miyamoto et al. 2016; Squarzoni et al. 2014; Ueno et al. 2013). This physiological task of microglia involves the selective elimination of synapses during development, through a highly coordinated process mediated by the fractalkine system known as ‘pruning’ (Paolicelli et al. 2011). Disruption of this process in mice in which the fractalkine receptor CX3CR1 has been deleted resulted in altered synaptic pruning, associated with weak synaptic transmission, decreased functional brain connectivity, as well as deficits in social interaction and increased repetitive behaviour (Zhan et al. 2014). These findings have contributed to an explosion of interest in the role of these cells in early development of the brain and especially in how disrupting this homeostatic function following exposure to early life perturbations (Bessis et al. 2007; Casano and Peri 2015; Ji et al. 2013; Kettenmann, Kirchhoff, and Verkhratsky 2013; Sierra, Tremblay, and Wake 2014; Wu et al. 2015) can subsequently lead to aberrant behaviours in the adult offspring (Ballas et al. 2009; Chen et al. 2001; Maezawa and Jin 2010; Paolicelli et al. 2011; Roumier 2004; Squarzoni et al. 2014; Zhan et al. 2014). To date much of this work has concentrated on testing environmental risk factors including stress, diet and maternal immune activation (MIA) in rodent models and their association with a number of neurodevelopmental disorders such as schizophrenia (SCZ) and autism spectrum disorder (ASD). Mechanistically, the focus of the majority of these studies has been on the integrity of the fractalkine system and how it is disrupted by MIA. Significant evidence (Mattei et al. 2017) in support of this has been put forward including some from our laboratory (Fernández de Cossío et al. 2017). In our study (Fernández de Cossío et al. 2017) and a number of others the pregnant animals were exposed

to the immunogenic stimulus in mid to late gestation, often at time points when microglial population of the fetal brain is complete and at the onset of synaptic pruning (Babri, Doosti, and Salari 2014; Bitanhirwe et al. 2010; Connor et al. 2012; Fernández de Cossío et al. 2017; Hsueh et al. 2017; Labrousse et al. 2018; Meyer 2006; Meyer et al. 2008; Zager, Menecier, and Palermo-Neto 2012). In other studies, the immunogen was administered much earlier in the gestation process (*e.g.* gestational day (GD)9, 9.5 or 10) at a time when microglia start to occupy the brain. This approach resulted in significant modifications in the behaviours of the offspring indicative of neurodevelopmental defects (Makinodan et al. 2008; Malkova et al. 2012; Meyer 2006; Meyer et al. 2008; da Silveira et al. 2017; Vuillermot, Feldon, and Meyer 2010).

Microglia have a unique origin, they arise from progenitors in the yolk sac, populating the developing brain through the blood following the establishment of the circulatory system, which in mice occurs from GD9.0 to GD10, and they are subsequently closed off from the periphery by the formation of the blood brain barrier at GD13.5 (Ginhoux et al. 2010, 2013; Kierdorf et al. 2013; Schulz et al. 2012). Importantly, these embryonically-derived microglia remain in the brain until adulthood (Ginhoux et al. 2010), highlighting the importance of the embryonic microglial development process as a period of high vulnerability for the fetal brain. This is based on the fact that microglia appear to be critical for the normal assembly of neuronal networks during early development. This was elegantly demonstrated by a study (Squarzoni et al. 2014) demonstrating that perturbation of microglial activity at the embryonic stage by using maternal lipopolysaccharide (LPS), a gram-negative bacteria endotoxin, affects the outgrowth of dopaminergic axons in the forebrain. Given that defects in dopamine innervation and cortical network formation have been linked to neuropsychiatric disorders adds credence to the notion that microglia contribute critically to the development of psychopathology. This study however did not delve into the effects on the changes affecting the microglia in this developmental stage. Using a similar approach to Squarzoni et al, we decided in the current investigation to assess whether an early immunogenic challenge will in any way alter the developmental trajectory of the microglia in the fetal brain and determine whether these will result in behavioural anomalies previously described in the offspring of MIA challenged mothers [see (Solek et al. 2018) for review]. For this we utilized

an MIA mouse model involving an early (GD9.5) injection of LPS to pregnant mice and performed analysis on the effects on microglial population across a number of embryonic developmental time points and how this was related to disrupted behaviours in the adult offspring.

## Materials and Methods

### *Timed Mating Procedure and Maternal Immune Activation*

Animal husbandry and experimental procedures were in accordance with the EU Directive 2010/63/EU for animal experiments and approved by the national ethical committee for care and use of animals (approval ID A13169). Male and female C57/BL6 mice of reproductive age (8 weeks old; Janvier Labs, Le Genest-Saint-Isle, France) were housed individually for ten days prior to breeding, to habituate to new housing conditions, under a 12hrs light/dark cycle (8am-8pm) with *ad libitum* access to food and water. Two females were then mated with one male for up to two days. Successful mating thereafter referred to as GD0.5, was verified the following morning by the presence of a vaginal plug. Pregnant females were weighed, individually housed and then injected at GD9.5 with either Saline (5 $\mu$ L/g) or LPS 125 $\mu$ g/kg (*Echerichia Coli* 0127:B8; lot #015M4041V; Sigma Aldrich) a gram negative component of the bacterial cell wall used to induce maternal immune activation. The resulting offspring were weaned and sexed at postnatal day (PND)21 and same-sex littermates housed together. Both male and female offspring were used in these and other studies as we have previously noted significant differences in some of the parameters measured along sex lines (Fernández de Cossío et al. 2017).

A cohort of Saline and LPS administered dams was sacrificed at either GD14.5 or GD18.5 to assess the effect of prenatal treatment on microglia cells in fetal brain (Saline: n=12 males/females, n=6 dams; LPS: n=12-14 males/females, n=6-7 dams). Another cohort of prenatally treated offspring underwent behavioural testing from PND3 to PND8 to assess reflexes, nest seeking behaviour, communication and isolation-induced communication (Saline: n=12-14 males/females, n=4 dams; LPS: n=15-16 males/females, n=5 dams). At adulthood (PND65-75), some of these animals were also tested for repetitive actions, social preference, social memory as well as amphetamine-induced locomotor behaviour (Saline: n=8-13 males/females, n=4 dams; LPS: n=7-10 males/females, n=5 dams).

### *Flow Cytometry Assay*

Offspring brains were collected at GD14.5 and GD18.5 and their sex determined by genotyping tail snips using established methods (McFarlane et al. 2013) (Saline: n=12

males/females, n=6 dams; LPS: n=12-14 males/females, n=6-7 dams). The collected tissues were dissociated into single-cell suspensions using a cell grinder. All cells were resuspended in buffer containing rat anti-mouse CD16/CD32 antibody (eBiosciences) to block nonspecific binding as previously described by us (Aguliar-Valles et al. 2014). Cells were then incubated with the following monoclonal antibodies: PE-conjugated anti-CD45 (1:100, eBiosciences), APC-conjugated anti-CX3CR1 (1:100, R&D Research) and PerCP-conjugated anti-CD117 (ckit) (1:100, eBiosciences). Staining was analyzed using a FACScalibur apparatus (BD Biosciences, Mississauga, ON, Canada) and FlowJo software.

### ***Righting and negative geotaxis reflex***

From PND3 to PND8, righting and negative geotaxis reflex were evaluated as described previously (Feather-Schussler and Ferguson 2016) (Saline: n=12-14 males/females, n=4 dams; LPS: n=15-16 males/females, n=5 dams). Reflexes were not measured at PND7 since nest seeking behaviour and ultrasonic vocalizations were assessed on this day. For the righting reflex, the newborns were placed on their back with their limbs pointing upwards and the time needed to turn over and restore their normal positions was recorded (maximum time was set at 30 seconds). Complete acquisition of reflex was assumed when the animals could rotate around its longitudinal axis and stand on all four limbs. For the negative geotaxis test, the offspring were placed on a 45° inclined surface, with their head facing downwards. Animals that could rotate a full 180°, face up, within a maximum time of 30 seconds were considered to have fully acquired this reflex.

### ***Ultrasonic vocalization***

Isolation-induced ultrasonic vocalizations (USVs) were measured following published protocols (Baharnoori, Bhardwaj, and Srivastava 2012; Grimsley, Monaghan, and Wenstrup 2011) (Saline: n=12-14 males/females, n=4 dams; LPS: n=15-16 males/females, n=5 dams). At PND7, the pups were placed alone in a sound attenuating chamber (20 cm x 15 cm) and their calls were recorded for 5 minutes. Bat detectors (Mini-3 Bat Detector; Ultra Sound Advice, London, UK) converted USVs into audible sounds. The output from the bat detector was fed into an adjustable audio filter, which then sent a predefined range of pitch and

loudness emitted by the pups to a computer equipped with Noldus UltraVox software (Version 1.0). The software recorded the time of the onset and offset of each vocalization and thus allowed the determination of the number and mean duration of USVs.

### ***Nest seeking behaviour***

The method was modified from existing nest seeking behaviour protocols also known as homing behaviour test (Antonelli et al. 2005; Baharnoori, Bhardwaj, and Srivastava 2012) (Saline: n=12-14 males/females, n=4 dams; LPS: n=15-16 males/females, n=5 dams). The animals were tested at PND7, 5 to 6 hours after the isolation-induced USVs measurement. The testing box consisted of a rectangular polycarbonate cage (42 x 26 x 18 cm) divided into 3 equal compartments: a central arena, a side compartment containing nest bedding from the test pup's home cage, and another side compartment on the opposite side containing the same quantity of fresh clean bedding. Each pup was placed in the central arena, crossing of the line toward nest compartment with the forepaws and head was considered a positive entry (cut-off time, 60 seconds). Each pup was assigned to 5 trials (inter-trial interval: 30 seconds, observation time: 60 seconds). The orientation in the center was also counterbalanced in-between trials.

### ***Three-Chamber Sociability test***

The three-chamber test was used during the dark phase to measure social preference and social memory as previously described by us (Fernández de Cossío et al. 2017) (Saline: n=8 males/females, n=4 dams; LPS: n=10 males/females, n=5 dams). Mice (PND65) were placed in a three-chamber plastic box (26 x 21.6 x 21.6 cm) with retractable doorways to allow entry in side-chambers. A wired container (9.5 x 7.6 cm) was placed in each of the two side chambers. The test had three consecutive phases: habituation, social preference and social memory. Habituation: the tested mouse was placed in the center chamber and allowed to explore the three chambers for 10 minutes. Social preference: the mouse was enclosed in the center compartment. A stranger mouse was placed in one of the wire cups while an object was placed under the other one. The doors were opened for a 10 minutes exploration. Social memory: the tested mouse was blocked in the middle chamber while the object was replaced

by a new stranger mouse. The doors to the side chambers were opened for 10 minutes. Stranger mice were age/sex-matched and habituated to the 3-chamber apparatus for 30 minutes the day before the test. A camera wired to an automated tracking system (SMART version 2.5.20; Panlab) was used to track the mice locomotion. Using the software, the experimental apparatus was divided in a central and two side chambers in which total time spent was analyzed.

### ***Marble burying test***

This was conducted as previously described by us (Fernández de Cossío et al. 2017), twelve glass marbles (15mm diameter) were placed equidistant on the surface of a 2.5cm layer of chipped wood bedding in a cage (28 x 17 x 12.7 cm). The tested mice (PND68) were placed in the cage and the number of marbles covered 2/3<sup>rd</sup> or more were counted after 30 minutes (Saline: n=8 males/females, n=4 dams; LPS: n=10 males/females, n=5 dams).

### ***Amphetamine-induced locomotor activity***

At PND75, Saline and LPS offspring' amphetamine-induced locomotion was assessed as previously described (Zager, Menecier, and Palermo-Neto 2012) (Saline: n=11-13 males/females, n=4 dams; LPS: n=7-9 males/females, n=5 dams). Mice were weighed and assigned to experimental cages (40 x 40 x 38 cm) where they were allowed to habituate for 30 minutes. Mice were then injected intraperitoneally (i.p.) with Saline solution (10 µL/g), and promptly placed back in their cage for 30 minutes. Finally, the mice were i.p. injected with amphetamine (2.5 mg/kg; 10 µL/g) and placed back in the cage for 1 hour. During the 3 phases of the test (Habituation, Saline and Amphetamine-induced locomotion), a camera wired to an automated tracking system (SMART version 2.5.20; Panlab) was used to track the mice locomotion.

### ***Statistical Analysis***

Two-way Analysis of Variance (ANOVA) with Tuckey post-hoc tests were used to assess the effect of treatment (Saline and LPS) and sex (Male and Female), as well as their potential interaction in Graphpad Prism 6 Software or SPSS. Two-way repeated measures ANOVA

with Tuckey post-hoc tests were used to assess the effect of treatment (Saline and LPS) and sex (Male and Female) and their potential interaction on latency during righting and geotactic reflex tests across age, and on locomotion during the amphetamine-induced locomotor activity test across time, using SPSS Software. All data are presented as mean values  $\pm$  standard error of the mean (SEM).

## Results

### *MIA alters microglial populations in the developing fetal brain.*

A refined methodology for characterizing microglia progenitors that can be observed in the yolk sac starting at GD7.5-8.0 was developed previously (Kierdorf et al. 2013). This study showed that CD45<sup>-</sup> ckit<sup>+</sup> erythromyeloid microglia progenitors mature into CD45<sup>+</sup> brain macrophages with a downregulation of the ckit marker, giving rise to two subsets of CD45<sup>+</sup> hematopoietic cells which are either immature (CX3CR1<sup>-</sup>) or mature (CX3CR1<sup>+</sup>) microglia at GD10.5 (Kierdorf et al. 2013). A more recent investigation (Matcovitch-Natan et al. 2016) identified a stepwise developmental program of microglia through three distinct stages - early, pre- and adult. Each stage in microglia development was found to be associated with different gene expression and functional stages that can be affected by environmental factors such as maternal immune activation. In our study, to examine the effect of early MIA on microglia cells during development, we injected pregnant mice with LPS at GD9.5 and collected fetal brains at GD14.5 (initiation of pre-microglia stage after early-microglia stage) and GD18.5 (pre-microglia stage) (Matcovitch-Natan et al. 2016). We used flow cytometry to gate the hematopoietic cell population (CD45<sup>+</sup>) (Figure III.1a & III.2a). Prenatal treatment had no effect on the percentage represented by hematopoietic cells among live cells at GD14.5 ( $F_{(1, 44)} = 0.846, p > 0.05$ ; Figure III.1d) and GD18.5 ( $F_{(1, 48)} = 2.569, p > 0.05$ ; Figure III.2d). In accordance with published characterization (Kierdorf et al. 2013) at GD14.5, the CD45 fraction could be further subdivided according to different levels of CX3CR1 expression, namely CX3CR1<sup>-</sup>, CX3CR1<sup>low</sup>, CX3CR1<sup>int</sup> (Figure III.1b). Also, as expected, the increased expression of CX3CR1 was concomitant with a downregulation of the hematopoietic stem cells marker ckit (Figure III.1c). Three sub-populations of microglia cells were distinguished in the fetal brain at GD14.5: CD45<sup>+</sup> CX3CR1<sup>-</sup> ckit<sup>+</sup> (immature), CD45<sup>+</sup> CX3CR1<sup>low</sup> ckit<sup>+/+</sup> (maturing) and CD45<sup>+</sup> CX3CR1<sup>int</sup> ckit<sup>+/+</sup> (mature). At GD18.5, only two subsets of CD45<sup>+</sup> hematopoietic cells were observed, either CX3CR1<sup>low</sup> (maturing) or CX3CR1<sup>int</sup> (mature) (Figure III.2b) and respectively ckit<sup>+/+</sup> and ckit<sup>-</sup> (Figure III.2c). At GD14.5, while prenatal treatment had no effect on the percentage of immature (CD45<sup>+</sup> CX3CR1<sup>-</sup> ckit<sup>+</sup>) and maturing (CD45<sup>+</sup> CX3CR1<sup>low</sup> ckit<sup>+/+</sup>) microglia, it significantly affected the percentage of mature microglia (CD45<sup>+</sup> CX3CR1<sup>int</sup> ckit<sup>+/+</sup>) among hematopoietic cells

(Immature:  $F_{(1, 44)} = 0.006$ ,  $p > 0.05$ ; [Figure III.1e](#); Maturing:  $F_{(1, 44)} = 1.459$ ,  $p > 0.05$ ; [Figure III.1f](#)) (Mature:  $F_{(1, 44)} = 5.055$ ,  $p = 0.029$ ; [Figure III.1g](#)). At GD18.5, prenatal administration of LPS also significantly affected the percentage represented by mature microglia cells ( $CD45^+ CX3CR1^{int} ckit^-$ ) among hematopoietic brain cells ( $F_{(1, 48)} = 19.55$ ,  $p < 0.001$ ; [Figure III.2e](#)). Indeed, *post-hoc* tests revealed a decreased percentage of mature microglia after LPS administration in both male and female fetuses (Male-Saline *vs.* Male-LPS:  $p = 0.007$ ; Female-Saline *vs.* Female-LPS:  $p = 0.041$ ).

### ***Maternal LPS alters behaviours in the neonatal and adult offspring.***

In order to test the effects of MIA on early/neonatal behaviour we performed both the righting and negative geotaxis reflexes to assess reflex development and neuromotor abilities from PND3 to PND8 in both male and female offspring of Saline and LPS treated dams. Pups from both treatment groups acquired these reflexes over a similar developmental time course (Righting reflex:  $F_{(1, 53)} = 0.074$ ,  $p > 0.05$ ; [Figure III.3a](#); Negative geotaxis reflex:  $F_{(1, 53)} = 0.082$ ,  $p > 0.05$ ; [Figure III.3b](#)). This observation is in accordance with results from our laboratory indicating the absence of gross neurological damage at PND4 in pups from dams having received an administration of LPS at GD15.0 (Fernández de Cossío et al. 2017). Age of the offspring had a significant effect on performance in the tests, reflecting the acquisition of basic motor reflexes (Righting reflex:  $F_{(4, 212)} = 62.104$ ,  $p < 0.001$ ) (Negative geotaxis reflex:  $F_{(4, 212)} = 36.413$ ,  $p < 0.001$ ). At PND7, olfactory discrimination of home bedding odor and nest seeking behaviour mediated by olfactory clues were tested with the nest seeking test. No effect of prenatal LPS administration was detected on either the number of entries in the nest compartment nor the latency to reach the compartment (Score:  $F_{(1, 53)} = 0.083$ ,  $p > 0.05$ ; [Figure III.3c](#)) (Latency:  $F_{(1, 53)} = 0.35$ ,  $p > 0.05$ ; [Figure III.3d](#)). Finally, in order to assess potential early deficits in communication, we measured the number of USVs emitted by pups when isolated from their mother and littermates, as well as the duration of their calls. While the number of USVs emitted was not affected by prenatal treatment ( $F_{(1, 53)} = 0.509$ ,  $p > 0.05$ ; [Figure III.3e](#)), the mean duration of these calls was significantly affected by prenatal administration of LPS ( $F_{(1, 53)} = 18.51$ ,  $p < 0.001$ ; [Figure III.3f](#)). As already shown by us previously using pregnant mice injected with LPS at GD15.0 (Fernández de Cossío et al.

2017), *post-hoc* analysis revealed that LPS-treated offspring emitted significantly shorter USVs (Male-Saline: 37.89 +/-9.537; Male-LPS: 28.13 +/-7.413; Female-Saline: 40.01 +/-6.875; Female-LPS: 29.13 +/-10.157; Male-Saline vs. Male-LPS:  $p=0.026$ ; Female-Saline vs. Female-LPS:  $p=0.017$ ).

To elucidate whether prenatal treatment had long-term functional consequences, we investigated the potential behavioural alterations in adult male and female offspring. Repetitive actions were measured using the marble burying test and were significantly affected by prenatal treatment ( $F_{(1, 32)} = 18.61$ ,  $p<0.001$ ; [Figure III.4a](#)). *Post-hoc* analysis further showed significantly altered behaviour in Male-LPS treated offspring with more repetitive actions and a trend in Female-LPS offspring (Male-Saline vs. Male-LPS:  $p=0.009$ ; Female-Saline vs. Female-LPS:  $p=0.073$ ). Social preference and social memory were assessed using the three-chamber test. During the habituation phase of the three-chamber test, no chamber preference was found in the experimental groups ([Supplementary Figure III.1](#)). However, both social preference and social memory were significantly affected by prenatal treatment ( $F_{(1, 32)} = 10.21$ ,  $p=0.003$ ; [Figure III.4b](#) and  $F_{(1, 32)} = 5.621$ ,  $p=0.024$ ; [Figure III.4c](#)). Sex had a significant effect on social preference ( $F_{(1, 32)} = 21.24$ ,  $p<0.001$ ) while a trend for sex effect on social memory was measured ( $F_{(1, 32)} = 2.957$ ,  $p=0.095$ ). *Post-hoc* analysis revealed that Male-LPS offspring displayed a significantly decreased social preference while specifically only Female-LPS offspring displayed a decreased social memory (Social preference - Male-Saline vs. Male-LPS:  $p<0.001$ ) (Social memory - Female-Saline vs. Female-LPS:  $p=0.004$ ). Finally, the potential impact of early MIA on amphetamine-induced locomotor activity as a behavioural indicator of abnormal activity in the mesolimbic dopaminergic pathway (Porrino et al. 1984) was assessed. During the habituation phase, locomotor activity was not affected by prenatal treatment ( $F_{(1,35)} = 0.008$ ,  $p>0.05$ ; [Figure III.4d](#)) or sex ( $F_{(1,35)} = 1.159$ ,  $p>0.05$ ; [Figure III.4d](#)), indicating that there was no difference in the baseline activity for the experimental groups. After Saline injection, the same pattern as during the habituation phase was measured: locomotor activity was not affected by treatment ( $F_{(1, 35)} = 0.251$ ,  $p>0.05$ ; [Figure III.4e](#)) or sex ( $F_{(1, 35)} = 1.411$ ,  $p>0.05$ ; [Figure III.4d](#)). Finally, after the amphetamine injection, time and sex significantly affected locomotor activity (Time effect:  $F_{(11, 385)} = 4.619$ ,  $p<0.001$ ; Sex effect:  $F_{(1, 35)} = 16.481$ ,  $p<0.001$ ; [Figure](#)

III.4f). Interestingly, while male and female offspring had similar locomotion during baseline and saline phases, after amphetamine administration, females had an overall increased locomotion (1481.987 +/- 108.650 vs. 1173.435 +/- 109.615). In accordance with results obtained in our laboratory on a rat model of MIA (Fortier et al. 2004), treatment had a significant effect ( $F_{(1, 35)} = 6.483$ ,  $p=0.011$ ) showing an overall increase in locomotion in LPS-treated offspring (1393.032 +/- 121.016 vs. 1261.967 +/- 95.792). A trend for an interaction between treatment and sex was measured ( $F_{(1, 47)} = 3.618$ ,  $p=0.058$ ), highlighting, once again, sexually dimorphic behavioural consequences triggered by MIA.

## Discussion

In the present study, we demonstrated that administration of LPS at an early stage of pregnancy does not affect the percentage of microglia in the embryonic brain but results in a microglial population with an immature phenotype. In addition, we assessed potential LPS-induced behavioural alterations in the offspring with a large battery of behavioural tasks and showed that while MIA does not affect reflex development, neuromotor ability and olfactory discrimination, it alters social/communicative behaviour in neonatal offspring. In adulthood, we measured significant effects of prenatal LPS on the offspring' repetitive behaviour, social preference, social memory and amphetamine-induced locomotor activity, most of which appeared to be prominent in the male progeny suggesting sexual dimorphism.

A primary objective of the current study was to further characterize the experimental approach used in our experiments. The use of MIA as a way of investigating the environmental impact on the developing fetal brain has expanded tremendously with a number of immunogens used including live viruses and viral mimics such as Poly(I:C) as well as bacteria and bacterial products, most notably LPS [see (Boksa 2003) for review]. In addition to these immunogens we have also developed a localized aseptic inflammation protocol using turpentine oil in pregnant mice (Aguilar-Valles et al. 2012). Whilst previous studies from our laboratories have utilized a number of these approaches, we have recently focused on the use of LPS. This was primarily driven by the consistency, reproducibility of the responses as well as the relative inflammatory potency activated when compared to for example Poly(I:C) (Fortier, Luheshi, and Boksa 2007). The other important variable that was considered in conducting these studies is the timing of the immunogen administration. Regardless of the immunogens used this varied considerably between laboratories and in some case even within the same laboratory including ours (Aguilar-Valles et al. 2012; Aguilar-Valles and Luheshi 2011; Ashdown, Dumont, et al. 2006; Fernández de Cossío et al. 2017; Fortier, Luheshi, and Boksa 2007; Fortier, Jooper, et al. 2004; Fortier, Kent, et al. 2004; Labrousse et al. 2018). In general, timing is considered a critical factor when studying inflammatory responses, especially when investigating the transient nature of changes in cytokine expression and release. This is particularly important during pregnancy when the

profile of the inflammatory mediators, cytokines, changes from a pro- to an anti-inflammatory setting with pregnancy progression (Aguilar-Valles et al. 2007; Ashdown, Poole, et al. 2006; Burns et al. 2015), which interestingly was suggested to be influenced by the sex of the developing fetus in human pregnancy (Burns et al. 2015). Thus, it was important for the purpose of this study to challenge the pregnant mother at a relatively early time point when pro-inflammatory cytokine activity is optimal and the anti-inflammatory influence is minimal. This allowed us to address the main objective of our investigation and that is to perform detailed analysis of microglial development and function and how this is influenced by the immunogen induced cytokine production. To achieve this therefore, we injected pregnant mice with LPS at GD9.5 (a time point characterized by migration of microglial progenitors in the rudimentary brain) and assessed microglia number and phenotype at GD14.5 [(end of early-microglia stage (Matcovitch-Natan et al. 2016)] and GD18.5 [pre-microglia stage (Matcovitch-Natan et al. 2016)]. A previous study (Kierdorf et al. 2013) addressed the question of the origin of microglia progenitors in the mouse embryo and identified the *ckit*<sup>+</sup> erythromyeloid progenitors as the earliest yolk sac microglia precursors. In the embryonic brain, these precursors then develop into mature microglia cells with a decreased expression of *ckit* (Kierdorf et al. 2013). A subsequent study measured levels of several microglia maturation factors and notably characterized microglia with an immature phenotype having increased expression of CSF1R, F4/80, CD31 (Matcovitch-Natan et al. 2016), and other surface molecules strongly developmentally regulated with decreasing levels during microglial maturation concomitant with *ckit* expression regulation (Kierdorf et al. 2013). In line with these characterizations of microglial populations across development, in this study, we noted distinct microglial populations in the developing fetal brain. These populations were characterized by a decreasing expression of *ckit* that was concomitant with an increasing expression of CX3CR1 as microglia matured. Importantly, we showed that prenatal LPS administration induces an overall immature phenotype of microglial cells. The functional consequences of these types of changes was clearly demonstrated in a study using germ-free mice, which exhibited global defects in microglia with an immature phenotype (Erny et al. 2015) resulting in a strongly diminished immune response of these cells in case of LPS immune challenge at adulthood. Matcovitch-Natan's

et al., (Matcovitch-Natan et al. 2016) identification of developmental program for microglial population in early brain development was based on differential genes regulation of microglia cells in the developing brain to acquire different functions. Interestingly, they showed that environmental perturbations of these pathways, with prenatal administration of the viral mimic Poly(I:C), can disrupt stage-specific functions of microglia; Poly(I:C)-treated offspring exhibiting microglia with an adult-like phenotype at GD14.5. In our experiments, prenatal administration of LPS, an immunogen that triggers similar inflammatory pathways, resulted in a decreased percentage represented by mature microglial population having low or undetectable levels of *ckit*. Interacting with its ligand, *ckit* triggers a chain of signaling events that activate downstream signaling pathways leading to cell proliferation (Antonchuk et al. 2004; Bernstein et al. 1991). Its expression progresses from low levels on primitive, dormant multipotent progenitors to high levels on later, actively cycling progenitors, and finally decreases to very low or undetectable levels on the most mature blood cells (Katayama et al. 1993). Thus, prenatal administration of LPS might affect microglia maturation, increasing the pool of immature microglia progenitors ( $CX3CR1^{low} ckit^{-/+}$ ) having important self-renewal/proliferative capacity. Ultimately, it could result in an increased number of microglia, a microglial dysregulation observed in post-mortem brains of patients with neurodevelopmental disorders (Morgan et al. 2010; Tetreault et al. 2012). The mature microglial population affected by prenatal LPS administration is also the population with the higher expression level of *CX3CR1* when compared to the maturing and/or immature populations. Fractalkine/*CX3CR1* signaling governs several functions of microglial cells in the developing brain such as neuronal survival (Ueno et al. 2013). Moreover, *CX3CR1* play a major role in determining how microglial cells influence the maturation of synaptic circuits during development by favoring microglia attraction at synaptic sites (Paolicelli et al. 2011; Rigato et al. 2011; Squarzoni et al. 2014). Furthermore, mice lacking the chemokine receptor *CX3CR1* exhibit a deficit in synaptic pruning that is associated with behavioural alterations (Zhan et al. 2014). We therefore consider that MIA affecting the maturation of the microglial population may perturb their physiological functions in the developing brain leading to long-term behavioural consequences.

We assessed the potential behavioural alterations in LPS-treated offspring with an array of behavioural tests. In agreement with clinical studies on children with developmental disorders, our model shows important features of neurodevelopmental disorders, while reflexes and gross motor skills remain unaltered (Ozonoff et al. 2008; Provost, Lopez, and Heimerl 2007; Rogers, Hepburn, and Wehner 2003). One of the most recognized tests to evaluate social communication is USVs (Ferhat et al. 2016). Indeed, these distress calls are emitted by the pups following maternal isolation to elicit maternal intervention. They can be affected in a number of complex ways, which may indicate altered emotional, social or communicative behaviour (Moy and Nadler 2008). In line with our previous results (Fernández de Cossío et al. 2017) and those of others (Baharnoori, Bhardwaj, and Srivastava 2012) we measured a decreased number of USVs emitted by LPS-treated offspring following separation from their mother during the neonatal period. In separate studies conducted at adulthood, we measured repetitive behaviours in Control and LPS-treated offspring. As with many investigations of this type, our model of prenatal administration of LPS results in an increase of this type of behaviours (Fernández de Cossío et al. 2017; Hsiao et al. 2012; Malkova et al. 2012; Onore et al. 2014; Schwartzer et al. 2013). We also assessed the effect of prenatal treatment on social preference and social memory. LPS-treated offspring displayed decreased social preference and social memory as measured by us and others in different rodent models of MIA (Aavani et al. 2015; Fernández de Cossío et al. 2017; Malkova et al. 2012; Onore et al. 2014; Xuan and Hampson 2014).

An interesting observation emanating from our studies is the clear separation of behavioural effects along sex lines. Sexual dimorphism is a recognized feature of neurodevelopmental disorders [see (Hanamsagar and Bilbo 2016b) for review], more often than not with a clear bias towards male vulnerability. In this study, it is interesting to note that while emission of USVs is decreased in both males and females LPS-treated offspring during neonatal period, at adulthood, the behaviours assessed are altered in a sex-specific manner. This onset of sex-specific behavioural alterations is in accordance with many neurodevelopmental diseases following a clear developmental pattern in addition to sex differences in prevalence or outcome [see (Hanamsagar and Bilbo 2016a) for review], that have been attributed, in large part, to the direct and indirect immuno-modulatory actions of sex hormones (Bouman, Jan

Heineman, and Faas 2005; Gaillard and Spinedi 1998; Klein 2000; Sakiani, Olsen, and Kovacs 2013). In this study, we did not assess the effect of MIA on adult microglia however, many other studies reported sex differences in microglia number and phenotype in many brain regions at adulthood (Hui et al. 2018; Lenz et al. 2013; Mouton et al. 2002; Schwarz, Sholar, and Bilbo 2012), and suggested that the interaction between microglia and sex hormones might play a pivotal role. Altogether, puberty appears as a critical period for microglial and behavioural sexualization. Indeed, many behaviours including social and repetitive behaviours are sensitive to gonadal hormones, the concentrations of which rise during puberty, occurring between PND26 and PND30 in mice [see (Bell 2018) for review]. For example, a recent study showed that males castrated before puberty (PND25) had greater anxiety-related behaviour during late puberty compared to intact males (PND40) (Boivin et al. 2017). Lenz et al. (Lenz et al. 2013) investigated microglial sex differences in the preoptic area and their influence in developmental masculinization. They showed that treating females with estradiol masculinize microglial number and morphology, toward amoeboid morphology. Moreover, microglial inhibition with minocycline prevented sex differences in microglia, estradiol-induced masculinization of microglial cells and copulatory behaviour. Altogether, they demonstrated that immune cells in the brain interact with the endocrine systems during development and are crucial for sexual differentiation of brain and behaviour. Such interaction between microglia and sexual hormones might explain the sexual dimorphism observed on behaviours at adulthood in LPS-treated animals.

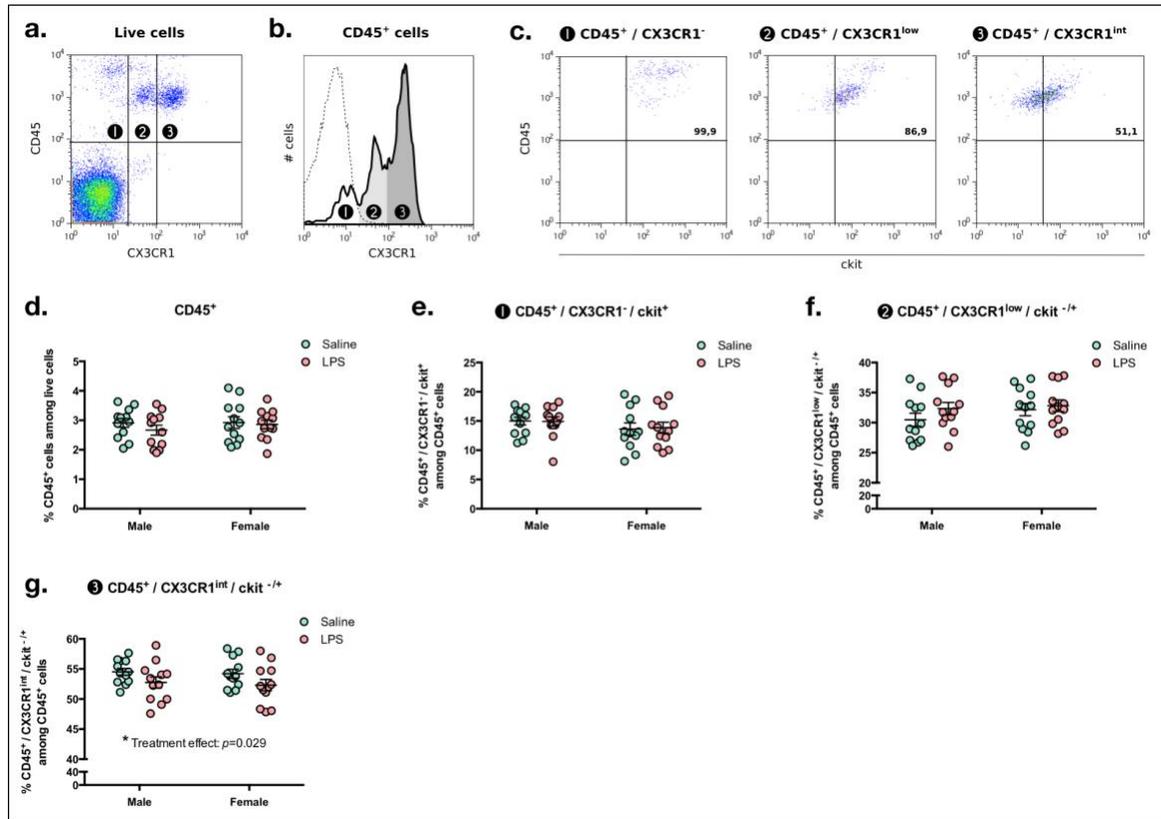
In summary, our studies showed that MIA at early stages of pregnancy results in microglial alterations during development as well as behavioural changes in the neonatal period and at adulthood. In order to assess potential effects of MIA on microglia cells in the adult brain and the association between microglial perturbation and behavioural alterations we conducted correlative studies on adult offspring detailed in the next chapter (Chapter 4).

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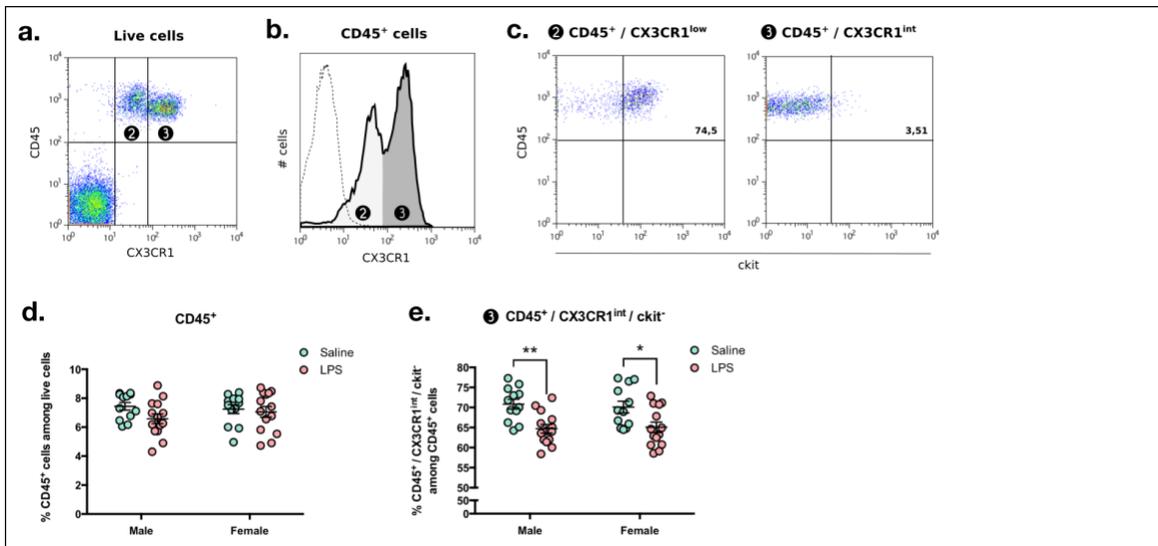
## Figures

**Figure III.1. Prenatal LPS administration at GD9.5 alters microglial cells maturation at GD14.5.**



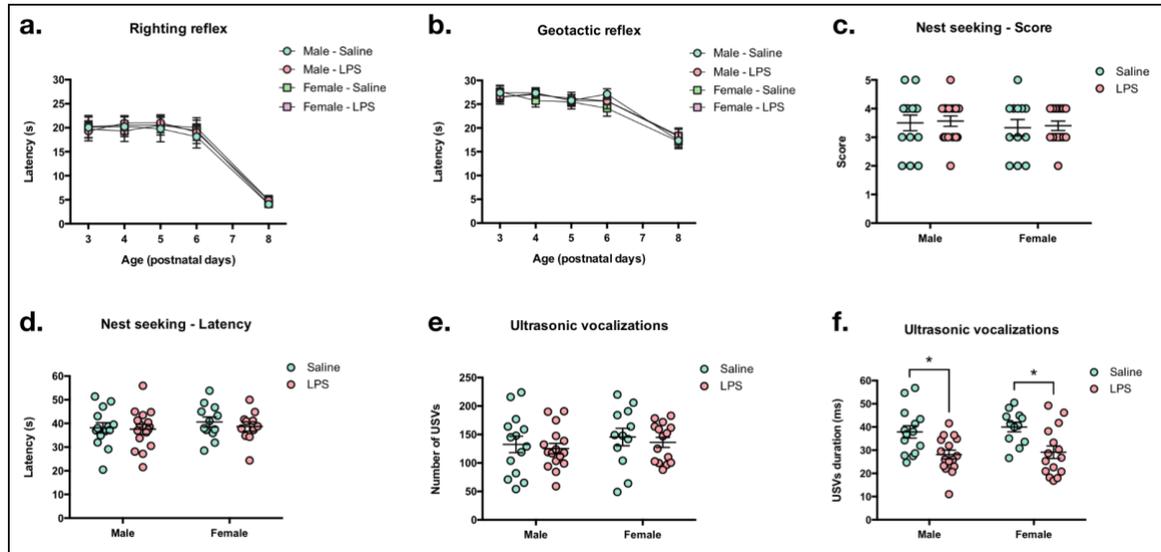
**Figure III.1.** Representative graphs of flow cytometry experiments (a-c). Fractionation of GD14.5 brain cells using CD45 and CX3CR1 markers (b) allowed for distinction of separate subsets of microglia cells: 1 CD45<sup>+</sup> / CX3CR1<sup>-</sup>, 2 CD45<sup>+</sup> / CX3CR1<sup>low</sup> and 3 CD45<sup>+</sup> / CX3CR1<sup>int</sup> that are respectively ckit<sup>+</sup>, ckit<sup>-/+</sup> and ckit<sup>-/+</sup> (c). Prenatal administration of LPS didn't affect the percentage of hematopoietic cells (CD45<sup>+</sup>) among live cells (d), nor the percentage represented by 1 CD45<sup>+</sup> / CX3CR1<sup>-</sup> / ckit<sup>+</sup> (e) and 2 CD45<sup>+</sup> / CX3CR1<sup>low</sup> / ckit<sup>-/+</sup> populations among hematopoietic cells (f) but significantly reduced the percentage represented by 3 CD45<sup>+</sup> / CX3CR1<sup>int</sup> / ckit<sup>-/+</sup> microglia population (g). (Saline: n=12 males/females, n=6 dams; LPS: n=12 males/females, n=6 dams). Data displayed represent the mean value  $\pm$  SEM (\* $P < .05$ ).

**Figure III.2. Prenatal LPS administration at GD9.5 alters microglial cells maturation at GD18.5.**



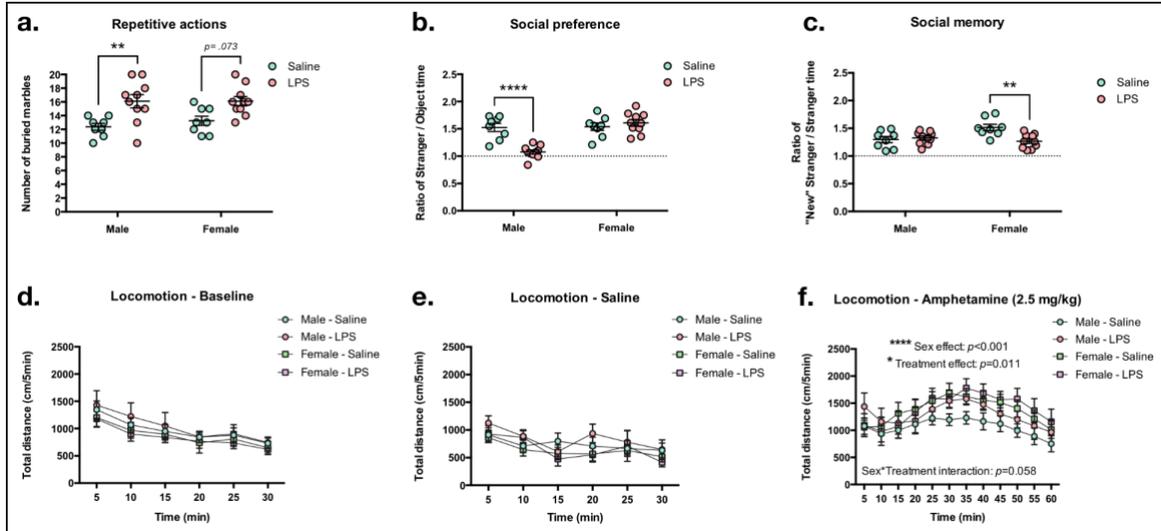
**Figure III.2.** Representative graphs of flow cytometry experiments (a-c). Fractionation of GD18.5 brain cells using CD45 and CX3CR1 markers (b) allowed for distinction of separate subsets of microglia cells: ② CD45<sup>+</sup> / CX3CR1<sup>low</sup> and ③ CD45<sup>+</sup> / CX3CR1<sup>int</sup> that are respectively ckit<sup>+/+</sup> and ckit<sup>-</sup> (c). Prenatal administration of LPS didn't affect the percentage represented by hematopoietic cells (CD45<sup>+</sup>) among live cells (d). At GD18.5, prenatal LPS significantly decreased the percentage represented by ③ CD45<sup>+</sup> / CX3CR1<sup>int</sup> / ckit<sup>-</sup> microglia population among live cells (e). (Saline: n=12 males/females, n=6 dams; LPS: n=14 males/females, n=7 dams). Data displayed represent the mean value  $\pm$  SEM (\* $P < .05$ , \*\* $P < .01$ ).

**Figure III.3. Prenatal LPS administration at GD9.5 alters communication behaviour but not neurological reflexes and nest-seeking behaviour measured during the neo-natal period.**

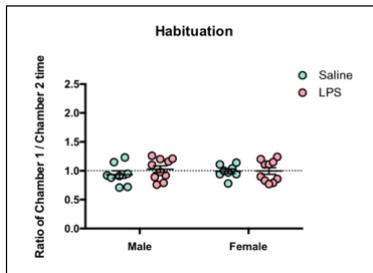


**Figure III.3.** Righting (a) and negative geotaxis (b) reflexes were measured from PND3 to PND8. These neurological reflexes were not affected by prenatal treatment. Nest seeking behaviour was measured at PND7 (c-d). The number of entries in the nest compartment (c) as well as the latency to reach this compartment (d) were not affected by prenatal LPS administration. Early life communication behaviour was assessed measuring isolation-induced ultrasonic vocalizations (USVs) at PND7 (e-f). Number of USVs emitted for 5 minutes isolation (e) was not affected by prenatal treatment. Mean duration of USVs was significantly decreased by prenatal LPS (f). (Saline:  $n=12-14$  males/females,  $n=4$  dams; LPS:  $n=15-16$  males/females,  $n=5$  dams). Data displayed represent the mean value  $\pm$  SEM (\* $P < .05$ ).

**Figure III.4. Prenatal LPS administration at GD9.5 affects repetitive actions, social preference, social memory and amphetamine-induced locomotion measured at adulthood with clear sexual dimorphism.**



**Figure III.4.** Prenatal administration of LPS induced increased repetitive actions in Male as measured via the marble burying test. The three-chamber test was used at PND65 to measure social preference (b) and social memory (c). Social preference was decreased in male offspring prenatally exposed to LPS while social memory was decreased in female-LPS offspring. A ratio of 1.0 (marked by a dotted line) denotes that equal time was spent with the object as with the stranger mouse or with the stranger as with the “new” stranger. Amphetamine-induced locomotor activity in adult offspring was measured (d-f). Locomotor activity under basal conditions (d) and 30 minutes after Saline injection (e) was not affected by prenatal treatment. Amphetamine-induced locomotor activity was affected by both prenatal treatment and sex (f). Activity is shown in 5 minute windows. (Saline: n=8-13 males/females, n=4 dams; LPS: n=7-10 males/females, n=5 dams). Data displayed represent the mean value  $\pm$  SEM (\* $P$ <.05, \*\* $P$ <.01, \*\*\* $P$ <.001, \*\*\*\* $P$ <.0001).

**Supplementary figure III.1.**

**Supplementary figure III.1.** *Habituation phase of the Three-chamber test assessed potential side chamber preference. (Saline: n=8 males/females, n=4 dams; LPS: n=10 males/females, n=5 dams). Data displayed represent the mean value  $\pm$  SEM.*

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**Chapter IV:**  
**Interleukin-1 $\beta$  mediates the effects of prenatal LPS induced  
behavioural anomalies in the offspring through modulating  
microglia**

## **IV.1. Preface**

In the previous chapter (Chapter 3), we demonstrated a clear effect of maternal LPS on the microglial population during embryonic brain development and behavioural anomalies in the adult offspring with clear sexual dimorphism. In this chapter, we will conduct detailed investigations to identify the potential underlying mechanism involved by focusing on the role of pro-inflammatory mediators, most notably IL-1 $\beta$ , a critical modulator of microglial activity. Our studies will also expand on our previous findings and those of others to elucidate whether the underlying mechanisms regulating changes in microglial population are also responsible for the sexually dimorphic behaviours noted in the offspring of prenatally challenged mothers.

## **IV.2. Manuscript**

**Title: Interleukin-1 $\beta$  mediates the effects of prenatal LPS induced behavioural anomalies in the offspring through modulating microglia**

**Chloé Lacabanne<sup>1,2</sup>, Lourdes Fernández de Cossío<sup>1</sup>, Sophie Layé<sup>2\*</sup> and Giamal N. Luheshi<sup>1\*</sup>**

<sup>1</sup> Douglas Hospital Research Centre, Department of Psychiatry, McGill University, Quebec, Canada H4H 1R3

<sup>2</sup> Nutrition et Neurobiologie Intégrée, UMR 1286, INRA, Université de Bordeaux, Bordeaux, France

\*Equal contribution

## Abstract

The role of microglia in orchestrating early neurodevelopmental processes has become an area of intense interest because of the reported association between disruption of this mechanism and the development of psychiatric disorders. Normal microglial function during embryonic development of the fetal brain has been shown to be vulnerable to environmental risk factors such as maternal infection. In previous studies, we and others have demonstrated that an immunogenic (bacterial or viral) maternal challenge results in offspring with abnormal behaviours, some of which have been associated with mental disorders in humans such as autism spectrum disorder (ASD) or schizophrenia (SCZ). The mechanisms, beyond the link with a general maternal inflammatory response, has not however been fully established. In the current investigation, we utilized an experimental approach of maternal immune activation (MIA) in mice at early stages of pregnancy using the bacterial cell wall product lipopolysaccharide (LPS), a potent activator of inflammatory mediators (cytokines) *in vivo*. The objective of this study was to identify the precise inflammatory mechanisms involved in disrupting microglial population during development *in utero* and the long-term behavioural consequences in the offspring.

Our results showed that LPS administration induced a significant inflammatory response exemplified by the induction of pro-inflammatory [interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF) $\alpha$ ] and anti-inflammatory (IL-10) cytokines in the maternal plasma, placenta and fetus. This was accompanied by an immature microglial phenotype at gestational day (GD)18.5 and a decreased percentage of microglia in brain at postnatal day (PND)9. These early alterations also resulted in long-term consequences including increased microglial density and changed morphology in specific brain structures as well as behavioural deficits, all of which were more prominent in the male progeny of inflamed mothers. Interestingly, the LPS-induced behavioural changes in adult mice correlated significantly with change in number of microglia in specific brain structures usually associated with these behaviours, supporting the involvement of microglial alterations in the development of these behaviours. Most interestingly, all of the LPS-induced changes demonstrated in this study were either prevented or significantly attenuated by the neutralization of maternal IL-1 $\beta$  using a

recombinant form of the naturally occurring IL-1 receptor antagonist (ra) co-administrated with the LPS challenge. This is the first evidence directly linking the activity of this cytokine with microglial changes *in utero*, which were extended to adulthood. This suggests a potential new avenue for therapeutic intervention in cases of mental disorders with a neurodevelopmental component triggered by a prenatal environmental challenge.

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## Introduction

The causes of neurodevelopmental disorders are poorly understood. The current most plausible theory indicates an interaction between genetic vulnerability and early environmental insults resulting in defective neuronal connectivity and subsequent pathology with schizophrenia (SCZ) (Blomström et al. 2016) and autism spectrum disorder (ASD) (Atladdottir et al. 2010) being prominent examples. Compared to the massive research effort dedicated to studying the genetics of neurodevelopmental disorders relatively little focus has been directed towards understanding mechanisms by which environmental risk factors contribute to the development of these conditions. Of the wide array of environmental factors that might play a role, there is considerable evidence to suggest that the intra-uterine environment is particularly influential. A meta-analysis found that several prenatal factors contribute to the risk for neurodevelopmental disorders such as ASD, including maternal gestational diabetes, maternal bleeding during pregnancy, and exposure to intrauterine infections (Gardener, Spiegelman, and Buka 2009). Several animal models have been developed to investigate the functional link between maternal infection and neurodevelopmental defects *in utero*, that leads to the development of psychiatric disorders in the progeny (Batinić et al. 2016, 2017; Cui et al. 2009; Fortier, Joobert, et al. 2004; Hsiao et al. 2012; Meyer 2006; Meyer et al. 2008). Whilst the majority of these studies have implicated a role for cytokines most notably interleukin (IL)-6 (Smith et al. 2007; W.-L. Wu et al. 2017), a precise mechanism of how these mediators affect brain development at the embryonic stage has remained elusive until recently. Indeed, new insights into the functions of microglia in the healthy developing brain in the mouse are providing a new perspective on this potential link [see (Salter and Stevens 2017) for review]. In addition to their physiological role of protecting the brain from injury and infection, microglia, the resident immune cells in the brain, are now known to contribute significantly to network formation and refinement by the elimination of superfluous synapses with a process known as “pruning” (Paolicelli et al. 2011). In recent studies testing this hypothesis and using a maternal immune activation model (MIA) we demonstrated a significant effect of the immunogenic challenge on microglial function and behaviour in the offspring (Fernández de Cossío et al. 2017). What triggers the transition of microglia from playing a homeostatic physiological role to a

pathological one that is detrimental to the developing brain is unknown, but we believe that it involves activation of these cells by maternal cytokines. Based on previous reports, the obvious cytokine is IL-6 (Smith et al. 2007; W.-L. Wu et al. 2017), however, to the best of our knowledge no one had demonstrated the expression of IL-6 receptors in microglia. Conversely, these microglia exhibit abundant expression of IL-1 receptors (Pinteaux et al. 2002). Indeed, microglia present one of the main targets of IL-1 $\beta$  in the brain, where it is expressed throughout development and for which, its upregulated expression is triggered by immunogenic challenges such as bacterial lipopolysaccharide (LPS) (Liu and Quan 2018a; Zaslona et al. 2017). Thus, in the present study we investigated the role of IL-1 $\beta$  in neurodevelopment using an MIA approach with LPS. These investigations were focused on early development [gestational day (GD)9.5], when microglia colonize the fetal brain (Ginhoux et al. 2010). Our rationale is that maternal challenge with LPS will induce the production of IL-1 $\beta$  in the maternal circulation and subsequently the fetal compartment disrupting the normal function of microglia and resulting in neurodevelopmental defects as recently described by others (Squarzoni et al. 2014; Thion and Garel 2017). Our studies were conducted in both males and females in order to further investigate the sexually dimorphic behaviour we have noted (Chapter 3), and how this relates to IL-1 $\beta$  activity and microglia during development.

## Materials and Methods

### *Timed Mating Procedure and Maternal Immune Activation*

All protocols were approved by McGill University's Animal Care Committee under the guidelines of the Canadian Council on Animal Care. Male and female C57/BL6 mice of reproductive age (8 weeks old; Charles River, Montreal, QC) were housed individually for ten days prior to breeding, to habituate to new housing conditions, under a 12hrs light cycle (8am-8pm) with *ad libitum* access to food and water. Two females were then mated with one male for up to two days. Successful mating thereafter referred to as GD0.5, was verified the following morning(s) by the presence of a vaginal plug. Pregnant females were weighed, individually housed and then injected at GD9.5 with either Saline (5 $\mu$ L/g) or Lipopolysaccharide (LPS) 125 $\mu$ g/kg (*Echerichia Coli* 0127:B8; lot #015M4041V; Sigma Aldrich) a gram negative component of the bacterial cell wall used to induce maternal immune activation. In rescue experiments, LPS was concurrently injected with recombinant human IL-1 receptor antagonist (ra) 100mg/kg (Kineret, SOBI, Sweden). In experiments where resulting offspring were used, pups were weaned and sexed at postnatal day (PND)21. Same-sex littermates were housed together in the housing conditions described previously. A cohort of pregnant mice was sacrificed 2 or 4 hours after prenatal Saline, LPS and LPS & IL-1ra treatment to assess the inflammatory response (Saline: n=5; LPS: n=5; LPS & IL-1ra: n=5). Another cohort of Saline, LPS and LPS & IL-1ra administered dams was sacrificed at either GD18.5 or PND9 to assess the effect of prenatal treatment on microglia cells in offspring' brain (Saline: n=18-20 males/females, n=9-10 dams; LPS: n=18 males/females, n=9 dams; LPS & IL-1ra: n=16-18 males/females, n=8-9 dams). Finally, a cohort of prenatally treated offspring was sacrificed at PND65 and their brain collected for immunohistochemistry (IHC) analysis (Saline: n=5 males/females, n=5 dams; LPS: n=5 males/females, n=5 dams; LPS & IL-1ra: n=5 males/females, n=5 dams) while another cohort underwent behavioural testing at adulthood before been sacrificed for flow cytometry experiments (Saline: n=8 males/females, n=8 dams; LPS: n=9 males/females, n=9 dams; LPS & IL-1ra: n=8 males/females, n=8 dams).

### ***Cytokine Assay***

Following anesthesia with rodent cocktail (Ketamine 100mg/mL, Xylazine 20mg/mL and Acepromazine 10mg/mL; 0.1mL/100g), pregnant mice were decapitated 2 or 4 hours after Saline, LPS or LPS & IL-1ra administration (at GD9.5) (Saline: n=5; LPS: n=5; LPS & IL-1ra: n=5). Trunk blood (collected in tubes containing 10µg of heparin) was centrifuged at 3600 rpm for 10min at 4°C. Plasma was then collected and stored at -80°C until analyzed. Placenta and fetuses were dissected immediately after decapitation of the mother and flash snap frozen, after which, they were homogenized in 500µL and 300µL of NP-40 Buffer respectively (150mM NaCl, 1% NP-40, 1M Tris-HCl and protease inhibitors). Total sample protein was determined using the BioRad DC Protein Assay kit (Lowry method) as per manufacturer's instructions. Levels of cytokines [IL-1β, IL-6, IL-10 and tumor necrosis factor (TNF)-α] in maternal plasma, placental and fetal tissues were measured with a Luminex Multiplex Assay (MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel, Millipore, Cat. #MPXMCYTO-70K) on a Luminex system according to the manufacturer's instructions. For all statistical analyses, values below the detection threshold of the assay were replaced with the minimal detectable value for the analyte (3.2pg/mL).

### ***Fluorescent Immunohistochemistry and Microscopy***

At PND65, offspring were anesthetized with rodent cocktail (0.3mL/100g) and perfused with 3.5% acrolein and 4% paraformaldehyde (Saline: n=5 males/females, n=5 dams; LPS: n=5 males/females, n=5 dams; LPS & IL-1ra: n=5 males/females, n=5 dams). Brains were removed and immersed in 4% paraformaldehyde for 2 hours, rinsed three times for 10 minutes with phosphate-buffered saline (PBS), transversally sliced with a vibratome (50µm thickness) and stored in cryoprotectant medium (30% ethylene glycol, 30% glycerol in PBS) at -20 °C before staining as previously described (Hui et al. 2018). Free-floating sections were immunostained using rabbit polyclonal anti Iba-1 (1:1000, #019-19741, Wako Chemicals GmbH) and goat anti-rabbit AlexaFluor 488 (1:1000, A11008, Invitrogen) as previously described in André et al (André et al. 2017). After washes in PBS and Triton 0.2%, sections were incubated in blocking solution (10% normal goat serum and 0.5% Triton X-100 in PBS) for 1h. Sections were incubated overnight with primary antibody at 4°C in

blocking buffer. After washes, sections were incubated with secondary antibody in blocking buffer for 2h, washed, mounted on slides in mounting medium with DAPI.

Using an Olympus VS120 Slide Scanner microscope, microglia were imaged in the prefrontal cortex, ventral hippocampus and amygdala of n=5 mice per experimental group with a 10x and 40x magnification for microglia density and morphology respectively. Z-stacks were acquired at 40x in an area covering the ventral hippocampus and contained ~30 z-planes (1µm each).

### ***Analyses of Microglial Density, Spacing and Morphology***

The numerical density of Iba1 cells (number cells/mm<sup>2</sup>) in the prefrontal cortex, ventral hippocampus and amygdala was determined for five sections per brain containing the areas of interest. The analysis was performed blind with ImageJ software (National Institutes of Health) as previously described (Hui et al. 2018; Tremblay et al. 2012). Briefly, the center of each microglial cell body was marked with the paintbrush tool. The cell numbers and their spatial coordinates were recorded using the “analyze particles” function. The nearest neighbor distance (NND) for each cell was then obtained with the “nearest neighbor distance” plugin. Cellular density was determined by dividing the total number of cells by the total surface area analyzed. The regularity index, describing the regular spacing of cells, was calculated as the ratio of the average NND of a population by the standard deviation of that population (Davis et al. 2017). A total of 15 microglial cells in the ventral hippocampus per animal were analyzed for microglia morphology. For each microglia, the soma area was determined by contouring the cell with the freehand selection tool. The arborization area was determined with the polygon selection tool to connect the most distal extremities of each process.

### ***Behavioural Testing***

The three-chamber and marble burying tests were performed to measure social preference, social memory and repetitive actions in adult offspring as previously described by us (Fernández de Cossío et al. 2017) (Saline: n=8 males/females, n=8 dams; LPS: n=9 males/females, n=9 dams; LPS & IL-1ra: n=8 males/females, n=8 dams). Briefly, the three-

chamber test had three consecutive phases: habituation, social preference and social memory. Habituation: the tested mouse was placed in the center chamber and allowed to explore the three chambers for 10 minutes. Social preference: the mouse was enclosed in the center compartment. A stranger mouse was placed in one of the wire cups while an object was placed under the other one. The doors were opened for a 10 minutes exploration. Social memory: the tested mouse was blocked in the middle chamber while the object was replaced by a new stranger mouse. For the marble burying test, twelve glass marbles (15mm diameter) were placed equidistant on the surface of a 2.5cm layer of chipped wood bedding in a cage. The tested mice (PND65) were placed in the cage and the number of marbles covered 2/3<sup>rd</sup> or more were counted after 30 minutes.

### ***Flow Cytometry Assay***

Offspring brains were collected at GD18.5, PND9 and PND70 (36h after the behavioural testing). Sex of GD8.5 fetus was determined by genotyping tail snips (McFarlane et al. 2013). For PND70, brain slices (1mm thick) were prepared using a chilled Mouse Brain Matrix from which the different structures were punched ([Supplementary Figure IV.1](#)). The collected tissues were dissociated into single-cell suspensions using a cell grinder. For PND9, the brain cell suspension was centrifuged with 37% Percoll (GE Healthcare Life Science) to remove myelin. All cells were resuspended in buffer containing rat anti-mouse CD16/CD32 antibody (eBiosciences) to block nonspecific binding as previously described by us (Aguilar-Valles et al. 2014). Cells were then incubated with the following monoclonal antibodies: FITC-conjugated anti-CD11b (1:100, eBiosciences), PE-conjugated anti-CD45 (1:100, eBiosciences), APC-conjugated anti-CX3CR1 (1:100, R&D Research) and PerCP-conjugated anti-CD117 (ckit) (1:00, eBiosciences). Staining was analyzed using a FACScalibur apparatus (BD Biosciences, Mississauga, ON, Canada) and FlowJo software.

### ***Statistical Analysis***

One and Two-way Analysis of Variance (ANOVA) with Tuckey post-hoc tests were used to assess the effect of treatment (Saline, LPS and LPS & IL-1ra) and sex (Male and Female) and their potential interaction in Graphpad Prism 6 Software. All data are presented as mean

values  $\pm$  standard error of the mean (SEM). Pearson correlation coefficient analysis was used to analyze the correlations between the % of microglia in each structure and the behavioural measurements on IBM SPSS Statistics software. A value of  $r$  ranging from 0.00-0.30 was regarded as “very weak”, 0.30-0.50 as “moderate”, 0.50-0.70 as “strong”, and 0.70-1.0 as “very strong” (Mukaka 2012). P-value less than 0.05 were deemed significant.

## Results

### *Co-administration of IL-1ra at the time of prenatal LPS injection regulates the MIA-induced cytokine production.*

In previous studies from our laboratories we demonstrated that LPS induces the production of pro and anti-inflammatory cytokines (mainly IL-1 $\beta$ , IL-6, TNF $\alpha$  and IL-10) in the plasma and brain of animals during neonatal period (Dinel et al. 2014) as well as in the maternal plasma, placenta and fetal brain following administration of LPS at GD18.0 (Ashdown, Dumont, et al. 2006; Labrousse et al. 2018). In the present investigation using a multiplex immunoassay panel, we measured IL-1 $\beta$  (Figure IV.1b), IL-6 (Figure IV.1c), TNF $\alpha$  (Figure IV.1d) and IL-10 (Figure IV.1e) in the maternal plasma, placenta, and whole fetus, 2 hours (left panels) and 4 hours (right panels) after administration of Saline, LPS or LPS + IL-1ra at GD9.5.

Under basal conditions, IL-1 $\beta$  (Figure IV.1b) was detected in both maternal plasma and fetus but not in the placenta. Following LPS administration, IL-1 $\beta$  expression was significantly induced in the maternal plasma (2hrs:  $F_{(2, 12)} = 6.478, p=0.012$ ; 4hrs:  $F_{(2, 12)} = 7.482, p=0.008$ ; Saline vs. LPS 2hrs:  $p=0.021$ ; 4hrs:  $p=0.008$ ), but not in the placenta and fetus (Placenta - 2hrs:  $F_{(2, 12)} = 1.233, p>0.05$ ; 4hrs:  $F_{(2, 12)} = 0.648, p>0.05$ ) (Fetus - 2hrs:  $F_{(2, 12)} = 0.448, p>0.05$ ; 4hrs:  $F_{(2, 12)} = 0.301, p>0.05$ ). IL-1ra co-administration prevented the LPS-induced IL-1 $\beta$  expression in the maternal plasma at 4 hours (Saline vs. LPS & IL-1ra:  $p>0.05$ ). IL-6 expression (Figure IV.1c) was significantly induced in the maternal plasma after LPS administration, while TNF $\alpha$  expression (Figure IV.1d) was significantly increased only 2 hours after LPS administration (IL-6 - 2hrs:  $F_{(2, 12)} = 34.13, p<0.001$ ; 4hrs:  $F_{(2, 12)} = 11.57, p=0.002$ ; Saline vs. LPS 2hrs:  $p<0.001$ ; 4hrs:  $p=0.002$ ) (TNF $\alpha$  -  $F_{(2, 12)} = 10.88, p=0.002$ ; Saline vs. LPS:  $p=0.002$ ). IL-6 was also significantly induced after LPS administration in both the placenta and the fetus (Placenta - 2hrs:  $F_{(2, 12)} = 94.02, p<0.001$ ; 4hrs:  $F_{(2, 12)} = 4.975, p=0.027$ ; Saline vs. LPS 2hrs:  $p<0.001$ ; 4hrs:  $p=0.022$ ) (Fetus - 2hrs:  $F_{(2, 12)} = 17.97, p<0.001$ ; 4hrs:  $F_{(2, 12)} = 5.566, p=0.019$ ; Saline vs. LPS 2hrs:  $p<0.001$ ; 4hrs:  $p=0.034$ ). Placental TNF $\alpha$  significantly increased after LPS administration (2hrs:  $F_{(2, 12)} = 17.93, p<0.001$ ; 4hrs:  $F_{(2, 12)} = 11.05, p=0.002$ ; Saline vs. LPS 2hrs:  $p<0.001$ ; 4hrs:  $p=0.004$ ), while TNF $\alpha$  was undetected

in the fetus regardless of time and treatment. IL-1ra co-administration prevented and/or significantly reduced LPS-induced placental IL-6 and TNF $\alpha$  (IL-6 - LPS vs. LPS & IL-1ra 2hrs:  $p=0.004$ ; Saline vs. LPS & IL-1ra 4hrs:  $p>0.05$ ) (TNF $\alpha$  - Saline vs. LPS & IL-1ra 2hrs & 4hrs:  $p>0.05$ ). IL-1ra also significantly prevented LPS-induced IL-6 in the fetus 2 and 4 hours after administration (Saline vs. LPS & IL-1ra 2hrs & 4hrs:  $p>0.05$ ). Basal levels of IL-10 (Figure IV.1e) were detected in all three compartments tested with high levels of expression particularly in the placenta, accordingly to previous studies (Lin et al. 1993). LPS significantly induced IL-10 concentration in the maternal plasma and the fetus 2 hours after administration (Maternal plasma -  $F_{(2, 12)} = 20.11$ ,  $p<0.001$ ; Saline vs. LPS:  $p=0.041$ ) (Fetus -  $F_{(2, 12)} = 9.147$ ,  $p=0.004$ ; Saline vs. LPS:  $p=0.014$ ). Importantly, LPS-induced IL-10 levels in the maternal plasma and fetus increased further in the presence of IL-1ra (Maternal plasma - LPS vs. LPS & IL-1ra 2hrs:  $p<0.05$ ; 4hrs:  $p=0.014$ ) (Fetus - LPS vs. LPS & IL-1ra 4hrs:  $p=0.27$ ). No significant effect of IL-1ra on IL-10 levels was observed in the placenta (2hrs:  $F_{(2, 12)} = 2.143$ ,  $p>0.05$ ; 4hrs:  $F_{(2, 12)} = 0.143$ ,  $p>0.05$ ).

***Co-administration of IL-1ra at the time of prenatal LPS injection regulates the effects of MIA on microglial population in the developing offspring's brain.***

The effect of MIA on cytokine expression in the fetus at GD9.5 prompted us to investigate the potential effects that these changes may have on microglia as these cells start to populate the brain at this early stage of development (Ginhoux et al. 2013) and are known to present a significant target of cytokines [see (Hanisch 2002) for review]. Giving the crucial role of microglia in onset and modulation of inflammation, it has been suggested that sex differences in microglial function could explain sexual dimorphism observed in MIA outcomes [see (Hanamsagar and Bilbo 2016) for review]. We thus assessed the effect of MIA on microglial population in both male and female offspring.

Using FACS analysis on whole fetal male and female brains at GD18.5 we demonstrated that the percentage represented by microglial population among live cells was not affected by treatment ( $F_{(2, 98)} = 2.245$ ,  $p>0.05$ ; Figure IV.2d). During early embryonic development (GD8.0), CD45<sup>-</sup> ckit<sup>+</sup> microglia progenitors observed in the yolk sac matured into CD45<sup>+</sup> head macrophages with a downregulation of ckit marker expression, giving rise to two

subsets of CD45<sup>+</sup> hematopoietic cells representing either immature (CX3CR1<sup>-</sup>) or mature (CX3CR1<sup>+</sup>) microglia at GD10.5 (Kierdorf et al. 2013). We distinguished two sub-populations of microglia cells: CD45<sup>+</sup> CX3CR1<sup>low</sup> ckit<sup>-/+</sup> (immature) and CD45<sup>+</sup> CX3CR1<sup>int</sup> ckit<sup>-</sup> (mature) (Figure IV.2a-c). A two-way ANOVA showed that the percentage of mature microglia (CD45<sup>+</sup> CX3CR1<sup>low</sup> ckit<sup>-/+</sup>) was significantly affected by prenatal treatment ( $F_{(2, 98)} = 26.77, p < 0.001$ ; Figure IV.2e). Indeed, *post-hoc* tests revealed a decreased percentage of mature microglia after LPS administration in both male and female fetal brain (Male-Saline vs. Male-LPS:  $p = 0.006$ ; Female-Saline vs. Female-LPS:  $p < 0.001$ ). This effect of LPS on the proportion of mature microglia was prevented by IL-1ra co-administration (Male/Female-Saline vs. Male/Female-LPS & IL-1ra:  $p > 0.05$ ). Similarly, postnatal analysis of microglial population at PND9 showed a significant effect of prenatal treatment on the percentage represented by microglial population within the offspring's brain ( $F_{(2, 106)} = 83.77, p < 0.001$ ; Figure IV.2f). *Post-hoc* analysis indicated that LPS exposed offspring had a lower percentage of microglia than Saline controls (Male-Saline vs. Male-LPS:  $p < 0.001$ ; Female-Saline vs. Female-LPS:  $p < 0.001$ ). At PND9, IL-1ra co-administration didn't prevent the effect of LPS on microglial population but significantly attenuated it: LPS & IL-1ra animals had a significantly higher percentage of microglia when compared to LPS treatment group (Male-LPS vs. Male-LPS & IL-1ra:  $p < 0.001$ ; Female-LPS vs. Female-LPS & IL-1ra:  $p < 0.001$ ), however, their percentage of microglia remained significantly lower than Saline control animals (Male-Saline vs. Male-LPS & IL-1ra:  $p < 0.001$ ; Female-Saline vs. Female-LPS & IL-1ra:  $p = 0.002$ ).

***Co-administration of IL-1ra at the time of prenatal LPS injection regulates the effects of MIA on microglial population in the brain of adult offspring.***

We further characterized the effect of MIA on microglia in different offspring's brain structures at adulthood (PND65), with specific interest in areas associated with higher-order cognitive functioning.

Prenatal treatment significantly affected microglial population in the Prefrontal cortex ( $F_{(2, 43)} = 26.81, p < 0.001$ ; Figure IV.3a), Ventral hippocampus ( $F_{(2, 43)} = 16.35, p < 0.001$ ; Figure IV.3b) and Amygdala ( $F_{(2, 43)} = 15.53, p < 0.001$ ; Figure IV.3c). *Post-hoc* analysis showed that

the percentage represented by microglia was increased in the Male-LPS and Female-LPS groups in the Prefrontal cortex (Male-Saline vs. Male-LPS:  $p < 0.001$ ; Female-Saline vs. Female-LPS:  $p = 0.003$ ) and in the Male-LPS group only in the Ventral hippocampus and Amygdala (Ventral hippocampus - Male-Saline vs. Male-LPS:  $p < 0.001$ ; Female-Saline vs. Female-LPS:  $p > 0.05$ ) (Amygdala - Male-Saline vs. Male-LPS:  $p < 0.001$ ; Female-Saline vs. Female-LPS:  $p > 0.05$ ). This sex specific LPS effect was attenuated by IL-1ra co-administration in the Ventral hippocampus (Male-Saline vs. Male-LPS & IL-1ra:  $p = 0.017$ ; Male-LPS vs. Male-LPS & IL-1ra:  $p = 0.014$ ) and completely abolished in the Prefrontal cortex and Amygdala (Prefrontal Cortex – Male/Female-Saline vs. Male/Female-LPS & IL-1ra:  $p > 0.05$ ) (Amygdala - Male-Saline vs. Male-LPS & IL-1ra:  $p > 0.05$ ). Prenatal treatment had no significant effect on percentage of microglia in other brain structures such as Somatosensory cortex, Dorsal hippocampus, Hypothalamus, Nucleus accumbens and Thalamus (Supplementary Figure IV.2). Finally, we confirmed this sex- and structure-specific LPS increase of microglia population that is prevented by IL-1ra co-administration with microglia density measurements on Iba1 immunostained brain images (Supplementary Figure IV.3) by assessing changes in various parameters previously used to characterize microglia phenotype (Hui et al. 2018; Tremblay et al. 2012). These included analysis of NND on Iba1 immunostained images which showed an effect of treatment in Prefrontal cortex ( $F_{(2, 24)} = 7.87$ ,  $p = 0.003$ ; Figure IV.3d), Ventral hippocampus ( $F_{(2, 24)} = 5.225$ ,  $p = 0.013$ ; Figure IV.3e) and Amygdala ( $F_{(2, 24)} = 7.502$ ,  $p = 0.003$ ; Figure IV.3f). These results parallel those obtained for density, given that as the number of microglia increases within a given area the space between the nearest cell should also decrease. Indeed, *post-hoc* analysis revealed significantly lower NND in Male LPS-treated offspring (Prefrontal Cortex - Male-Saline vs. Male-LPS:  $p = 0.015$ ) (Ventral hippocampus - Male-Saline vs. Male-LPS:  $p = 0.006$ ) (Amygdala - Male-Saline vs. Male-LPS:  $p = 0.004$ ). This LPS-induced NND decrease was no longer observable when IL-1ra was co-administrated with LPS (Male-Saline vs. Male-LPS & IL-1ra:  $p > 0.05$ ). The regularity index, which is defined as the ratio of the mean NND over the NND standard deviation and describes the regular spacing of cells (Davis et al. 2017), remained unchanged across experimental groups, in the three examined brain regions (Prefrontal cortex:  $F_{(2, 24)} = 3.570$ ,  $p > 0.05$ ; Figure IV.3g) (Ventral hippocampus:  $F_{(2, 24)} =$

0.913,  $p > 0.05$ ; **Figure IV.3h**) (Amygdala:  $F_{(2, 24)} = 0.158$ ,  $p > 0.05$ ; **Figure 3i**). Microglia density, NND and Regularity index were also measured in Somatosensory cortex with immunostained images and there was no difference across experimental groups (**Supplementary Figure IV.4**). To further study the effect of early MIA on microglia cells, morphological analyses were performed on microglia in the ventral hippocampus of adult offspring. Prenatal treatment significantly affected both cell body and arborization area ( $F_{(2, 24)} = 17.48$ ,  $p < 0.001$ ; **Figure IV.3j** and  $F_{(2, 24)} = 27.24$ ,  $p < 0.001$ ; **Figure IV.3k**). *Post-hoc* analyses revealed a significantly larger cell body area in LPS treated Female offspring and a trend in LPS Male (Female-Saline vs. Female-LPS:  $p = 0.003$ ; Male-Saline vs. Male-LPS:  $p = 0.069$ ) as well as a significantly smaller arborization area in LPS treated animals (Male-Saline vs. Male-LPS:  $p = 0.002$ ; Female-Saline vs. Female-LPS:  $p < 0.001$ ). Co-administration of IL-1ra prevented these effects of LPS with microglia from animals in this group not significantly different from the Saline controls (Male/Female-Saline vs Male/Female-LPS & IL-1ra:  $p > 0.05$ ). When a morphological index was computed by dividing soma area by arborization area (Hui et al. 2018; Tremblay et al. 2012), a ratio which is susceptible to increase in reactive microglia (Streit, Walter, and Pennell 1999), a treatment effect was also identified ( $F_{(2, 24)} = 52.96$ ,  $p < 0.001$ ; **Figure IV.3l**). Subsequent *post-hoc* analyses showed that adult LPS offspring had increased morphology index (Male-Saline vs. Male-LPS:  $p < 0.001$ ; Female-Saline vs. Female-LPS:  $p < 0.001$ ) while offspring in the LPS & IL-1ra group had indices that did not differ from those of the Saline group (Male/Female-Saline vs Male/Female-LPS & IL-1ra:  $p > 0.05$ ).

***Co-administration of IL-1ra at the time of prenatal LPS injection prevents the negative effects of MIA on the offspring' behaviour.***

In previous studies using prenatal LPS injected at GD14.5 we demonstrated significant effect on a number of behavioural paradigms tested in the offspring (Fernández de Cossío et al. 2017) which we subsequently confirmed in data presented in Chapter 2 using the earlier time point of injection during pregnancy (GD9.5). Here we focused in on social preference and social memory assessed with the three-chamber test and repetitive behaviour assayed with marble burying identified previously (Chapter 2) as reliable indicators of the LPS effect on

the offspring' behaviour. During the habituation phase of the three-chamber test, no chamber preference was found in the experimental groups (Supplementary Figure IV.5). However, prenatal treatment had a significant effect on both social preference and social memory ( $F_{(2, 43)} = 7.672, p=0.001$ ; Figure IV.4a and  $F_{(2, 43)} = 6.753, p=0.003$ ; Figure IV.4b), and *post-hoc* analysis revealed that these changes were specific to Male-LPS offspring (Social preference: Male-Saline vs. Male-LPS:  $p<0.001$ ) (Social memory: Male-Saline vs. Male-LPS:  $p=0.035$ ). This effect of LPS on male offspring was prevented by the prenatal IL-1ra co-administration (Male-Saline vs. Male-LPS & IL-1ra:  $p>0.05$ ). Prenatal treatment also had a significant effect on repetitive actions measured during the marble burying test ( $F_{(2, 43)} = 41.80, p<0.001$ ; Figure IV.4c). *Post-hoc* analysis further showed altered behaviour in the offspring of LPS treated mothers with more repetitive actions in both sexes (Male-Saline vs. Male-LPS:  $p<0.001$ ; Female-Saline vs. Female-LPS:  $p=0.009$ ). Similar to our findings with the three-chamber test, IL-1ra co-administration prevented the LPS-induced repetitive behaviour (Male/Female-Saline vs. Male/Female-LPS & IL-1ra:  $p>0.05$ ).

***MIA-induced changes in microglia in specific structures of the adult offspring' brain correlate significantly with behavioural disruptions.***

To analyze a potential correlation between microglia changes and behaviour, a correlation analysis was performed (Figure IV.5 and Table IV.1 for detailed  $r$  and  $P$ -value). These analyses revealed a strong negative correlation between social preference and percentage represented by microglia cells among live cells specifically in the Amygdala ( $r = -0.549, p<0.001$ ) and Ventral hippocampus ( $r = -0.567, p<0.001$ ) while it did not correlate with any other analyzed brain structure (Somatosensory cortex, Dorsal hippocampus, Hypothalamus, Nucleus accumbens and Thalamus). There was also a significant negative correlation between Social memory and proportion of microglia cells in Ventral hippocampus specifically ( $r = -0.521, p<0.001$ ). Interestingly, chamber preference measured during the habituation phase of the three-chamber test did not correlate with any microglia population measurement in brain structures ( $-0.3 < r < 0.3$ ). Repetitive actions strongly correlated with changes in percentages represented by microglia in the Amygdala ( $r = 0.523, p<0.001$ ), Prefrontal cortex ( $r = 0.650, p<0.001$ ) and Ventral hippocampus ( $r = 0.636, p<0.001$ ).

## Discussion

In the present study, we showed that LPS administration at early stages of pregnancy induces a significant inflammatory response in the maternal plasma, placenta and fetus, along with changes in the microglial population of the developing brain. These early alterations resulted in long-term consequences including increased microglial density and changed morphology in specific brain structures, as well as behavioural deficits, all of which were more prominent in the male progeny of infected mothers. Most importantly, we demonstrate that these changes are mediated by IL-1 $\beta$ . These observations indicate a strong functional association between MIA induced microglial impairment at the embryonic stage and altered behaviours in the adult offspring.

The developmental time point at which the mothers were exposed to LPS treatment (GD9.5), is the time when newly migrated microglia progenitors colonize the brain, go through maturation to then take on an array of developmental functions that can be affected by environmental factors such as MIA (Matcovitch-Natan et al. 2016). In line with results presented in the previous chapter (Chapter 2), we observed no effect of prenatal treatment on the percentage of microglia among live cells in the fetal brain at GD18.5; however, within this microglial population, we detected a significant decrease in the percentage of mature microglia in the LPS-treated offspring. In this study, we also investigated the potential long-term effects of prenatal LPS on microglia at postnatal period and adulthood. At PND9, the percentage of microglia among live cells in the whole brain was reduced in contrast to brains from PND65, which exhibited an increased percentage in specific brain structure (prefrontal cortex, ventral hippocampus and amygdala) of LPS-treated offspring. However, since we did not assess microglial population in the whole brain at adulthood, we cannot exclude a global decrease of this population at PND65 as measured at PND9. The significance of interrupting normal microglial maturity is not clear from our studies however, investigations from others suggest a link with reduced proliferative capacity and ultimately altered functionality (Erny et al. 2015).

Our findings showing LPS-induced microglial density changes in specific higher-order cognitive-emotional structures that are implicated in complex behaviours and neuropsychiatric disorders (prefrontal cortex, ventral hippocampus and amygdala) indicate that a functional relationship may exist with some behavioural outcomes in the offspring. To investigate this link, we performed a correlative study in which brains from animals used for the behavioural analysis were removed following the completion of the tests and analysis of microglial density conducted. These studies demonstrated a remarkable correlation between reduction in microglial density in the amygdala and the ventral hippocampus and behavioural outcomes in the adult mice of LPS treated mothers. These brain structure-specific correlations are consistent with previous research that characterized the structures/circuits implicated in social behaviour (Felix-Ortiz and Tye 2014; Gunaydin et al. 2014; Y. Kim et al. 2015). Several studies conducted in rodents and primates on the brain regions and circuits encoding or storing social memory (Okuyama 2018) highlighted the involvement of neural circuits in the ventral hippocampus. In line with these findings, we observed a strong correlation between social memory alterations and decrease of microglia density specifically in the ventral hippocampus. Similarly, increased repetitive actions correlated with LPS effects on microglia cells in the amygdala, prefrontal cortex and the ventral hippocampus, three main structures that form part of the circuit underlying repetitive behaviours in mouse models (H. Kim, Lim, and Kaang 2016). How changes in microglia affect the formation and function of these circuits was not investigated in the current study however they described key role in neurodevelopmental processes, including the establishment of viable neuronal connections and brain homeostasis [see (Arnold and Betsholtz 2013; Bilimoria and Stevens 2015; Frost and Schafer 2016; Mosser et al. 2017; Paolicelli and Ferretti 2017; Reemst et al. 2016; Y. Wu et al. 2015) for review] strongly suggest their involvement. In this regard, prenatal immune activation could act directly on microglia during development (altering their maturation and proliferation) altering their function in the developing brain, resulting in changing the developmental trajectories and ultimately leading to the behavioural and neuronal deficits detected in MIA models (Knuesel et al. 2014; Paolicelli and Ferretti 2017). In support of this hypothesis is the identification of a pro-inflammatory profile in the developing and neo-natal brain of LPS-treated offspring (O’Loughlin et al. 2017). Signs of

inflammatory processes as well as changes in microglia density and morphological anomalies were also observed in post-mortem brains of a subset of schizophrenic and ASD patients (Van Kesteren et al. 2017; Morgan et al. 2010; Vargas et al. 2005). Indeed, despite the variability across studies, there is a constant observation for increased microglial density (in accordance with our findings at adulthood) and morphological alterations with somal enlargement and process retraction in brain of patients diagnosed with neurodevelopmental disorders. In this study, in addition to the change in number of cells we performed morphological analysis using specific parameters described by Tremblay et al., (Tremblay et al. 2012) measuring notably soma and arborization area of microglia. In a recent study, we (Hui et al. 2018) demonstrated Poly(I:C) induced significant changes in microglia morphology in the dentate gyrus. Likewise, our work showed a significant effect of prenatal treatment on microglia morphology index, a ratio which is susceptible to increase in reactive microglia (Streit, Walter, and Pennell 1999), in adulthood. Altogether, it suggests a long-term priming/programming of microglia cells at early stages of development by MIA.

One of the main objectives of the current investigation was to try and identify the mechanisms responsible for altering the microglial development and function. Given the nature of the maternal challenge (LPS), it has always been assumed that immune activation is the key driver, and several studies including some from our own laboratory (Ashdown, Dumont, et al. 2006; Ashdown, Poole, et al. 2006; Cui et al. 2009; Luheshi 1998) have demonstrated a general increase in pro-inflammatory cytokines in the maternal plasma, placentas and fetal brains. Other studies have narrowed this down to providing evidence supporting a specific role for IL-6 (Hsiao et al. 2012; Smith et al. 2007; W.-L. Wu et al. 2017). These researchers used a neutralization strategy in the mother to demonstrate an attenuation of behavioural outcomes in the adult offspring, a powerful demonstration of the involvement of this cytokine. However, despite this, the exact mechanism regulated by IL-6 either in the mother or the developing fetus was not provided. It is unlikely that this cytokine is involved in modulating microglial activity as, to the best of our knowledge, these cells do not express IL-6 receptors. This is in contrast to IL-1 $\beta$  whose receptors are abundant on microglia (Pinteaux et al. 2002) and IL-1 $\beta$  is one of the main mediators of both immunological and non-immunological functions of these cells (Liu and Quan 2018b).

This role for IL-1 $\beta$  is unequivocally supported by our present data which demonstrates for the first time, that it is a critical mediator of LPS-induced alteration of microglial development in the fetal brain, the embryonic inhibition of which offers significant protection against the behavioural anomalies exhibited in the offspring of MIA challenged mothers. The majority of the evidence presented here supporting a role for IL-1 $\beta$  in the MIA-induced changes at both microglial and behavioural levels were obtained using intervention studies by utilizing the endogenously occurring IL-1ra antagonist. The benefits of using this inhibitor as a way of protecting a developing fetus was previously demonstrated in a prenatal model where LPS was injected at a late stage in pregnancy resulting in placental inflammation, significant cell death and fetal mortality all of which were attenuated or prevented by the co-administration of IL-1ra (Girard et al. 2010). It is important to note here that in contrast to the present study where IL-1ra was administered early in pregnancy (GD9.5), these researchers injected this antagonist at the end of gestation. This is an important consideration given that the timing of the injection of this inhibitor is determined principally by the changes in its endogenous circulating concentrations which during pregnancy is known to increase exponentially in tandem with gestation time (Graham et al. 2017). This is demonstrated in a very recent study where the late gestation administration of IL-1ra failed to protect against a number of fetal anomalies associated with preterm birth (Nadeau-Vallée et al. 2017) whilst the use of a non-competitive IL-1 receptor biased ligand did (Nadeau-Vallée et al. 2015). Nevertheless, the current investigation in addition to numerous other studies from our lab (Ashdown, Poole, et al. 2006; Fortier, Kent, et al. 2004; Miller, Hopkins, and Luheshi 1997; Pohl, Luheshi, and Woodside 2013) attest to the importance of this reagent as a tool for these types of investigations and its potential development as an effective therapeutic that could, potentially, be utilized as a preventative measure against neurodevelopmental disorders, in susceptible pregnant women. Indeed IL-1ra is already being used clinically for the treatment of rheumatoid arthritis and neonatal onset multisystem inflammatory disease [see (Dayer, Oliviero, and Punzi 2017; Rosenzweig, Lei, and Burd 2014) for review]. In addition to inhibiting IL-1 $\beta$  activity our results suggest that IL-1ra promotes the production of other anti-inflammatory cytokines, in this case IL-10. The importance of IL-10 itself has been demonstrated when it was shown to be a key determinant of pregnancy resilience to

inflammatory stress or more specifically infection with LPS (Rivera et al. 1998). Indeed, IL-10 modulates resistance to inflammatory stimuli by downregulating expression of pro-inflammatory cytokines such as TNF $\alpha$  and IL-6 in order to protect against inflammation-induced pathology in the implantation site (Robertson, Care, and Skinner 2007). This indicates a multiplicity in the activity of IL-1ra to further promote its anti-inflammatory activity.

Perhaps one of the most interesting outcomes of our current study is the clear sex biases towards the male progeny in almost all the parameters investigated. Sexual dimorphism is becoming an area of much interest in neurodevelopmental research and microglial function appears to be a central component regulating these differences (Lenz et al. 2013; Lenz and McCarthy 2015; Vanryzin, Pickett, and McCarthy 2018). Clinically the significance of this is that a variety of neurodevelopmental disorders including ASD and SCZ [see (Hanamsagar and Bilbo 2016) for review] are associated with sex differences in prevalence or symptomatology, with males 4-8 times more likely to be affected than females (Bale 2016). Although only a few studies directly compared the effects of MIA on behaviour in both male and female offspring, most, including our own studies (Fernández de Cossío et al. 2017; Hui et al. 2018) find a more severe behavioural deficit in males compared to females (Meehan et al. 2017; Naviaux et al. 2015; Taylor et al. 2012; Xuan and Hampson 2014). In MIA type studies investigating microglial responses either failed to report the use of male's vs females [e.g. (Cunningham, Martinez-Cerdeno, and Noctor 2013; Hsiao et al. 2012; Pratt et al. 2013; Roumier et al. 2008)] or used only males or a mix of males and females (e.g. (Van Den Eynde et al. 2014; Giovanoli et al. 2015; Hadar et al. 2017; Paylor et al. 2016)). Studies which did make that distinction reported substantial differences between male and female on microglial properties in basal conditions, including microglia density and morphology, phagocytic activity, migration (Nelson, Warden, and Lenz 2017; Schwarz, Sholar, and Bilbo 2012; Weinhard et al. 2018; Yanguas-Casás et al. 2018). In the current study, we demonstrate significant differences in the behaviours of male offspring when compared to female littermates from LPS treated mothers, including decreased social behaviour and social memory, when compared to the Saline groups. This is consistent with our previous findings

(Fernández de Cossío et al. 2017; Hui et al. 2018). In addition, we observed striking sexual dimorphism when investigating the effect of MIA on microglia cells at adulthood. FACS and immunohistochemical analysis demonstrated an increased percentage of microglia cells within the prefrontal cortex, ventral hippocampus and amygdala specifically in male progeny of LPS treated mothers. Multiple studies already reported sex differences in microglia number and phenotype in many brain regions at adulthood (Hui et al. 2018; Lenz et al. 2013; Mouton et al. 2002; Schwarz, Sholar, and Bilbo 2012) and suggested that the interaction between microglia and sex hormones might play a pivotal role. While we did not measure baseline sex difference in adult microglia density in the brain structures investigated in offspring from Saline treated mothers, it is interesting to note the switch from LPS effects observed during gestation and postnatal period, to Male-specific LPS effects observed at adulthood. Indeed, while we measured the same consequences of prenatal LPS administration on both Male and Female at GD18.5 and PND9, at adulthood, MIA effects measured in specific brain structures were predominantly different in males. This non-sex-specific effect of LPS on microglial density is in perfect accordance with a recent study assessing the impact of Poly(I:C) administration at GD15.0 on microglial immunoreactivity in several brain regions of the rat offspring at PND2 (Zhang et al. 2018). In line with other studies, our results also suggest a role of gonadal hormones in the LPS-induced effects and thus defines puberty as a critical period for behavioural sexualization. Indeed, many behaviours among which social and repetitive behaviours are sensitive to gonadal hormones the concentrations of which rise during puberty occurring between PND26 and PND30 in mice [see (Bell 2018) for review]. For example, a recent study showed that males castrated before puberty (PND25) had greater anxiety-related behaviour during late puberty compared to intact males (PND40) (Boivin et al. 2017). In addition, to the effect of sexual hormones during puberty, “fetal programming” that makes one sex more vulnerable to environment and/or hormone levels over the other should be considered. In this regard, the placenta has recently become a target of interest. A recent and exciting hypothesis has suggested the involvement of the placenta in producing sex-specific transcriptional response as well as relaying sex-specific transplacental signals to the developing brain in case of changes or perturbations in the maternal milieu (Bale 2016). Interestingly, male placenta has been shown

to be a great deal more responsive to environmental stressors, including infection, than female counterparts (Charil et al. 2010; Davis and Pfaff 2014; Gabory et al. 2013; Howerton and Bale 2012; Nugent and Bale 2015; Prins et al. 2018; Sandman, Glynn, and Davis 2013).

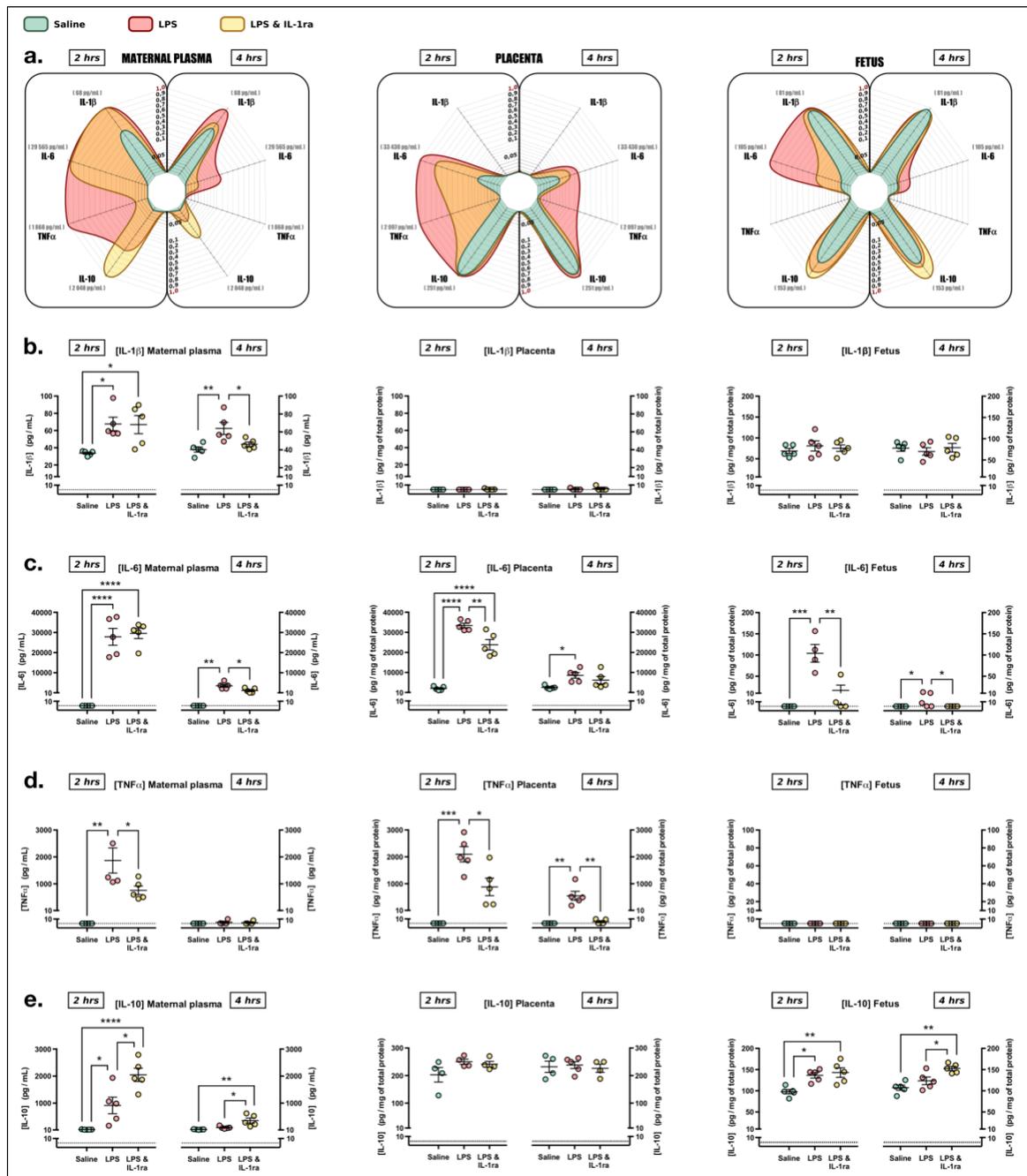
In summary, in this study we show that through an IL-1 $\beta$ -driven mechanism, maternal inflammation during pregnancy leads to severe and long-term effects on both microglia cells and behaviour. Importantly, the strong correlations between structure-specific changes in microglia density and specific behavioural alterations suggests that prenatal LPS effects on microglia might play an important role in neurodevelopmental disorders. Moreover, the sexual dimorphism observed in LPS-induced microglial and behavioural alterations at adulthood suggests a sex-specific “fetal programming” of microglia cells that could be due to a differential response to LPS determined by placental sex. Lastly, we highlight the use of IL-1ra as a potential preventive therapeutic in mothers experiencing an early environmental challenge of a known risk factor such as infection.

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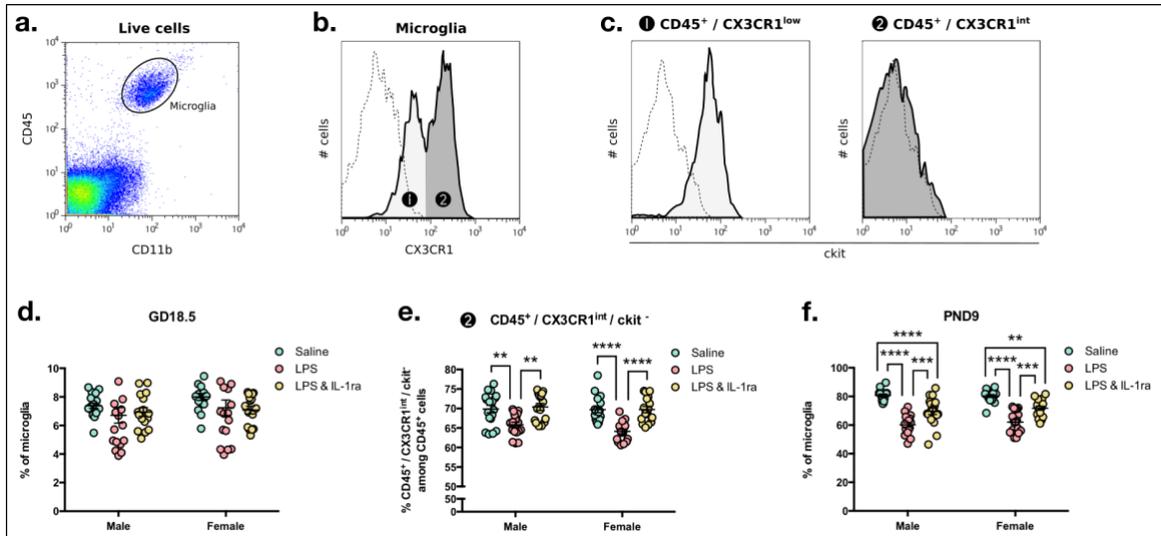
## Figures and Tables

**Figure IV.1. Multiplex analysis of pro and anti-inflammatory cytokines in maternal plasma, placenta, and fetus 2 and 4 hours after prenatal LPS and IL-1ra co-administration.**



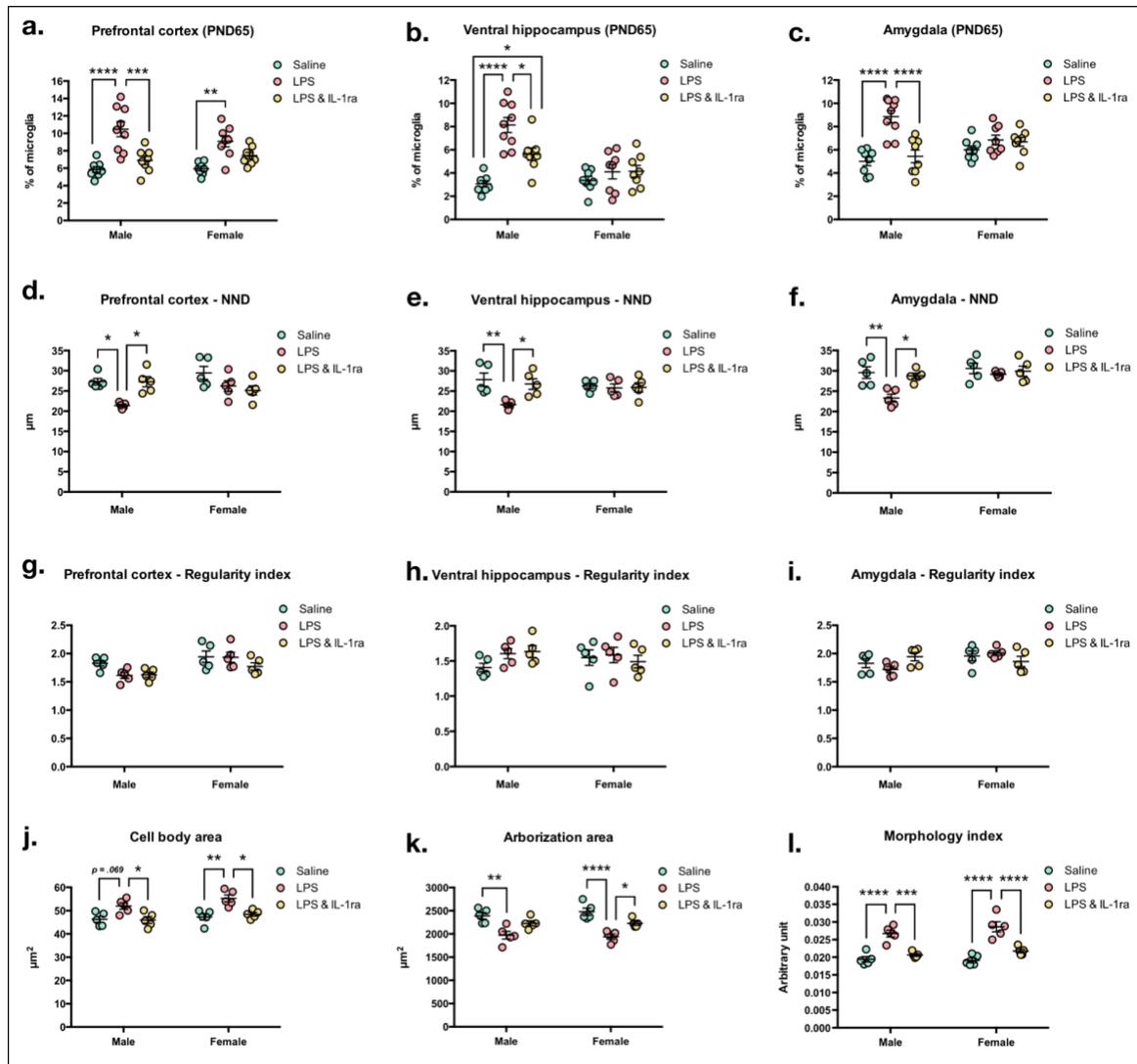
**Figure IV.1.** Radar chart representation of cytokine concentration in maternal plasma (left), placenta (middle) and fetus (right), 2 and 4 hours after Saline, LPS, or LPS & IL-1ra administration at GD9.5 (a). The polygons' perimeter displays the mean value per group, as a percentage of the maximum concentration for each cytokine in a given compartment, at one timepoint. Concentration level of IL-1 $\beta$  (b), IL-6 (c), TNF $\alpha$  (d) and IL-10 (e) for each treatment group in maternal plasma, placenta and fetus. The dashed line represents the detection limit (3.2 pg per mL or per mg of total protein) of the immunoassay. Multiplex analysis was performed on the plasma of N=5 pregnant dams (with > 7 fetus) per experimental group. Data displayed represent the mean value for each individual pregnant dam or litter  $\pm$  SEM (\* $P$ <.05, \*\* $P$ <.01, \*\*\* $P$ <.001, \*\*\*\* $P$ <.0001).

**Figure IV.2. Prenatal co-administration of IL-1ra prevents LPS effects on microglia maturity at GD18.5 and attenuates its impact on total microglia number at PND9.**



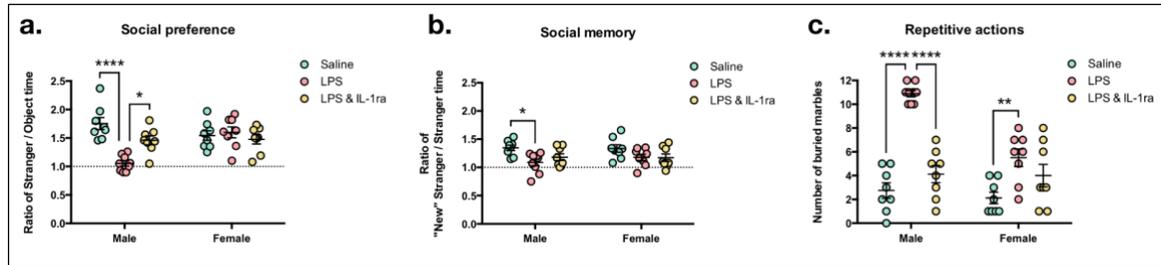
**Figure IV.2.** Representative graph of flow cytometry experiments (Male-Saline GD18.5 embryo) (a-c). Fractionation of GD18.5 brain cells using CD45 and CD11b markers allowed the distinction of microglia cells population (a). Within the CD45<sup>+</sup> / CD11b<sup>+</sup> cell population (microglia) (b) two subsets were found: 1 CX3CR1<sub>low</sub> (immature) and 2 CX3CR1<sup>int</sup> (mature) that are respectively c-kit<sup>+/+</sup> and c-kit<sup>-</sup> (c). Prenatal LPS administration didn't affect the total percentage of microglia cells among live cells at GD18.5 (d), but significantly decreased the percentage represented by 2 CD45<sup>+</sup> / CX3CR1<sup>int</sup> / ckit<sup>-</sup> mature microglia among total microglia population regardless of the sex (e). This effect was prevented by IL-1ra co-administration (e). (Saline: n=18 males/females, n=9 dams; LPS: n=18 males/females, n=9 dams; LPS & IL-1ra: n=16 males/females, n=8 dams). Prenatal LPS administration resulted in a decreased percentage of microglia cells within live cells in brains from animals at PND9 (f). This effect was attenuated when IL-1ra was co-administered with LPS (f). (Saline: n=20 males/females, n=10 dams; LPS: n=18 males/females, n=9 dams; LPS & IL-1ra: n=18 males/females, n=9 dams). Data displayed represent the mean value  $\pm$  SEM (\*P<.05, \*\*P<.01, \*\*\*P<.001, \*\*\*\*P<.0001).

**Figure IV.3. Prenatal co-administration of IL-1ra prevents LPS-induced microglia density and morphology alterations in male offspring at PND65.**



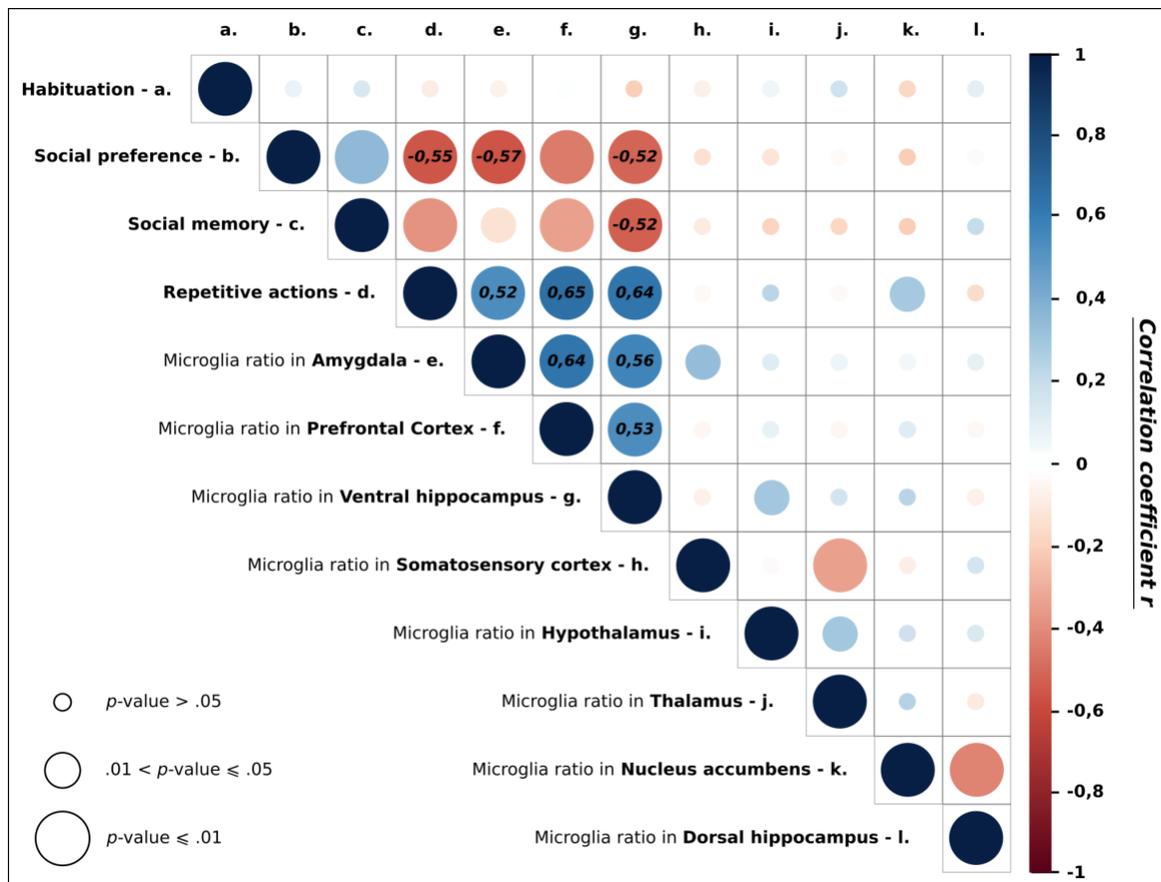
**Figure IV.3.** Flow cytometry analysis of CD45 and CD11b microglia cells of the prefrontal cortex (a), ventral hippocampus (b) and amygdala (c) of PND65 offspring 36 hours following the 3-chamber and the marble burying tests. (Saline: n=8 males/females, n=8 dams; LPS: n=8-9 males/females, n=8-9 dams; LPS & IL-1ra: n=8 males/females, n=8 dams). Analysis of nearest neighbor distance (NND) (d-f) and resulting regularity indices (g-i) were performed on Iba1 immunostained images of all three structures tested. Cell body area (j) and arborization area (k) resulting in morphological index (l) were measured in the ventral hippocampus. Cell body area of microglia was significantly increased in the ventral hippocampus of LPS Female animals and IL-1ra co-administration abrogated this effect (j). Prenatal LPS administration significantly decreased the arborization area regardless the sex of the offspring (k). (Saline: n=5 males/females, n=5 dams; LPS: n=5 males/females, n=5 dams; LPS & IL-1ra: n=5 males/females, n=5 dams). Data displayed represent the mean value  $\pm$  SEM (\* $P$ <.05, \*\* $P$ <.01, \*\*\* $P$ <.001, \*\*\*\* $P$ <.0001).

**Figure IV.4. Prenatal co-administration of IL-1ra prevents LPS-induced alterations in social preference, social memory and repetitive behaviour at PND65 in a sex-specific manner.**

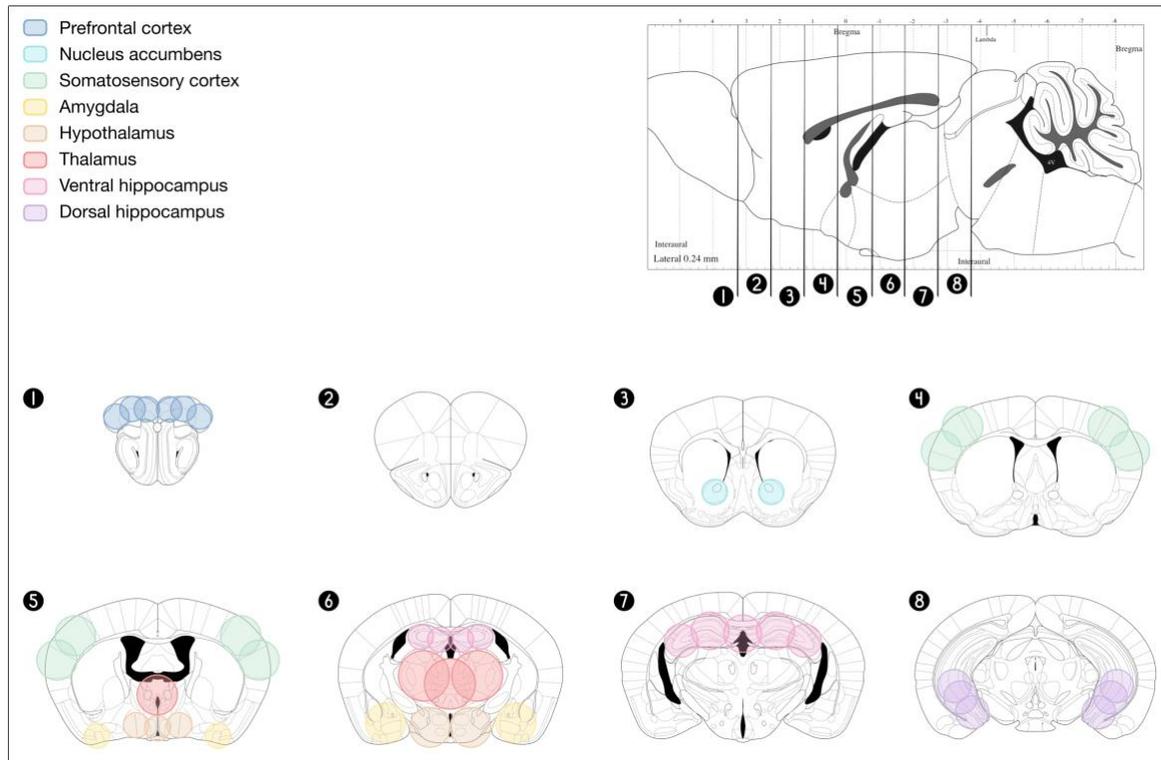


**Figure IV.4.** Social preference (a) and social memory (b) measured using the three-chamber test were altered in male offspring prenatally exposed to LPS and were prevented by the co-administration of IL-1ra. A decreased ratio of 1.0 (marked by a dotted line) denotes that equal time was spent with the object as with the stranger mouse or with the stranger as with the "new" stranger. Prenatal administration of LPS induced increased repetitive actions in both males and females as measured by the marble burying test (c). This effect was prevented by the co-administration of IL-1ra. (Saline:  $n=8$  males/females,  $n=8$  dams; LPS:  $n=8-9$  males/females,  $n=8-9$  dams; LPS & IL-1ra:  $n=8$  males/females,  $n=8$  dams). Data displayed represent the mean value  $\pm$  SEM (\* $P<.05$ , \*\* $P<.01$ , \*\*\* $P<.001$ , \*\*\*\* $P<.0001$ ).

**Figure IV.5. Behavioural alterations correlate with increased percentage of microglia cells in specific brain structures.**

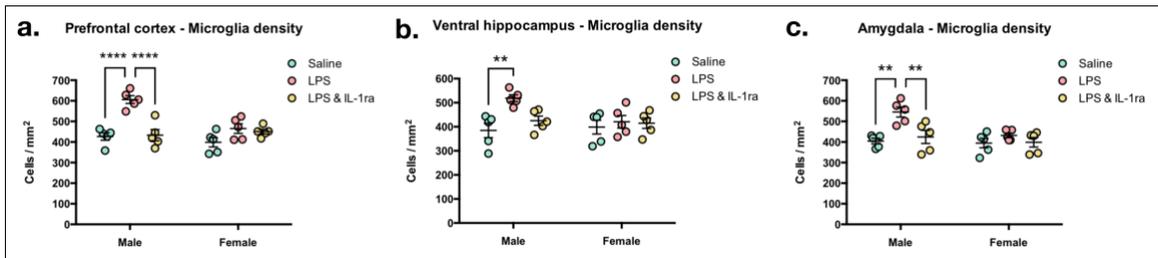


**Figure IV.5.** Positive correlation (blue), no correlation (white), and negative correlation (red). Pearson coefficient is displayed only when the correlation is considered “strong” ( $r > 0.5$ ). (Saline:  $n=8$  males/females,  $n=8$  dams; LPS:  $n=8-9$  males/females,  $n=8-9$  dams; LPS & IL-1ra:  $n=8$  males/females,  $n=8$  dams). Size of circles indicates significance of correlation ( $*P < .05$ ,  $**P < .01$ ,  $***P < .001$ ).

**Supplementary figure IV.1.**

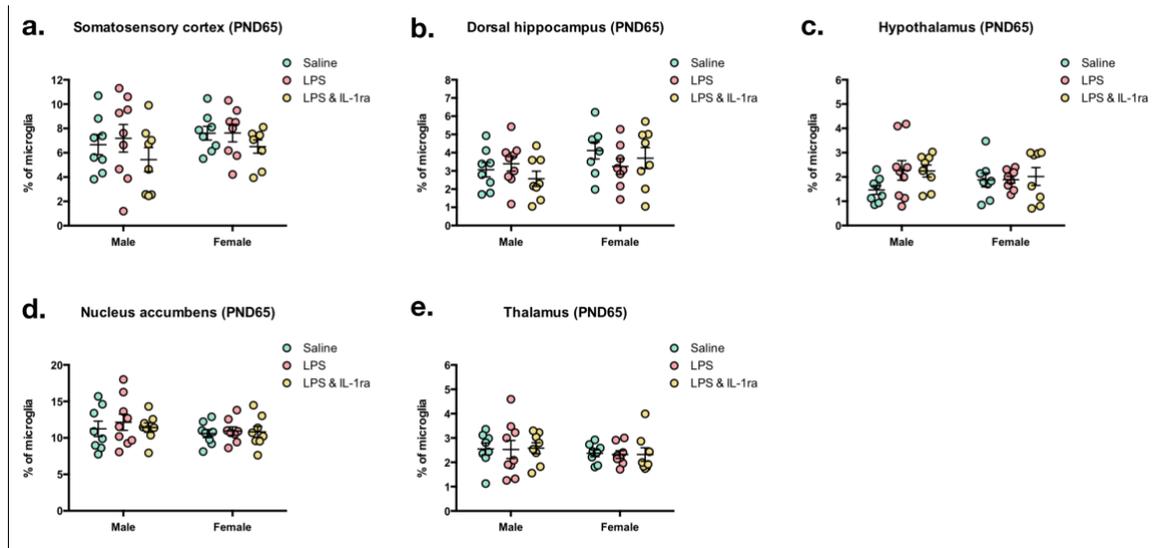
**Supplementary figure IV.1.** *The shaded circles/areas mark sites where the brain tissue was dissected out with 1 mm or 2 mm diameter micro punches. The Prefrontal cortex, Nucleus Accumbens, Somatosensory cortex, Amygdala, Hypothalamus, Thalamus, Ventral and Dorsal hippocampus were punched from brain coronal sections. Brain sections are mapped onto plates adapted from *The Mouse Brain in Stereotaxic Coordinates atlas* (Konsman 2003) (1 to 8) at +3.20, +2.22, +1.18, +0.38, -0.22, -1.22, -2.18, -2.16 mm from Bregma.*

## Supplementary figure IV.2.

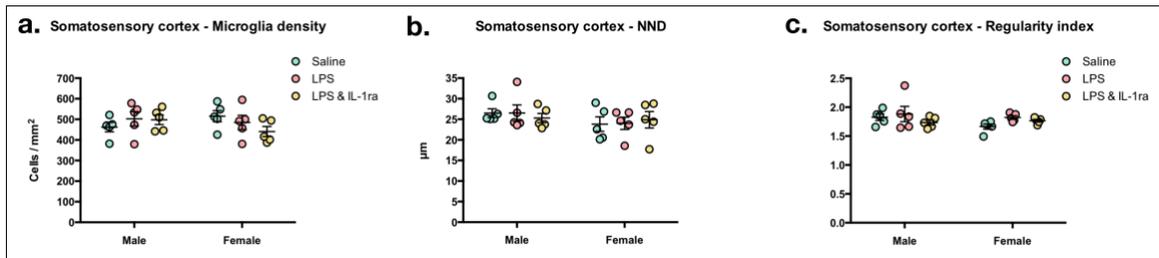


**Supplementary figure IV.2.** Quantitative analysis on *Iba1* immunostained 10x images of prefrontal cortex, ventral hippocampus and amygdala to assess the density of microglia (a-c). Density of microglia cells was significantly increased in prefrontal cortex (a), ventral hippocampus (b), and amygdala (c) of PND65 male offspring prenatally exposed to LPS, an effect that was prevented by the co-administration of IL-1ra. (Saline: n=5 males/females, n=5 dams; LPS: n=5 males/females, n=5 dams; LPS & IL-1ra: n=5 males/females, n=5 dams). Data displayed represent the mean value  $\pm$  SEM (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$ ).

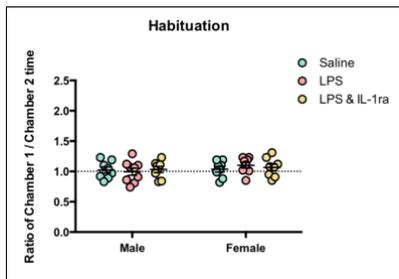
## Supplementary figure IV.3.



**Supplementary figure IV.3.** *CD45* and *CD11b* markers were used to distinguish the microglia cell populations within somatosensory cortex (a), dorsal hippocampus (b), hypothalamus (c), nucleus accumbens (d) and thalamus (e) of PND65 offspring. Flow cytometry was performed on microglia of mice 36 hours following the 3-chamber and the marble burying tests. (Saline:  $n=8$  males/females,  $n=8$  dams; LPS:  $n=8-9$  males/females,  $n=8-9$  dams; LPS & IL-1ra:  $n=8$  males/females,  $n=8$  dams). Data represent the mean value  $\pm$  SEM.

**Supplementary figure IV.4.**

**Supplementary figure IV.4.** Quantitative analysis on *Iba1* immunostained 10x images of somatosensory cortex of PND65 offspring exposed to prenatal treatment to assess the density (a), nearest neighbor distance (NND) (b) and regularity index of microglia cells (c). (Saline:  $n=5$  males/females,  $n=5$  dams; LPS:  $n=5$  males/females,  $n=5$  dams; LPS & IL-1ra:  $n=5$  males/females,  $n=5$  dams). Data displayed represent the mean value  $\pm$  SEM.

**Supplementary figure IV.5.**

**Supplementary figure IV.5.** *Habituation phase of the Three-chamber test assessed potential side chamber preference. (Saline: n=8 males/females, n=8 dams; LPS: n=8-9 males/females, n=8-9 dams; LPS & IL-1ra: n=8 males/females, n=8 dams). Data displayed represent the mean value  $\pm$  SEM.*

**Table IV.1.**

Correlation coefficient	a.	b.	c.	d.	e.	f.	g.	h.	i.	j.	k.	l.
a. Habituation	1	0,082	0,132	-0,078	-0,047	0,008	-0,202	-0,060	0,048	0,172	-0,175	0,091
b. Social preference		1	0,352**	-0,549***	-0,567***	-0,454***	-0,521***	-0,129	-0,184	-0,082	-0,213	0,008
c. Social memory			1	-0,389**	-0,324*	-0,340**	-0,521***	-0,093	-0,193	-0,170	-0,217	0,207
d. Repetitive actions				1	0,523***	0,650***	0,636***	-0,018	0,228	-0,016	0,291	-0,144
e. Microglia ratio in Amygdala					1	0,635***	0,564***	0,326*	0,100	-0,063	0,042	0,094
f. Microglia ratio in Prefrontal Cortex						1	0,527***	-0,034	0,078	-0,034	0,107	-0,028
g. Microglia ratio in Ventral Hippocampus							1	-0,063	0,297*	0,150	0,221	-0,056
h. Microglia ratio in Somatosensory Cortex								1	-0,002	-0,339**	0,072	0,161
i. Microglia ratio in Hypothalamus									1	0,298*	0,111	0,134
j. Microglia ratio in Thalamus										1	0,23	-0,096
k. Microglia ratio in Nucleus accumbens											1	-0,420**
l. Microglia ratio in Dorsal Hippocampus												1

**Table IV.1.** Correlation matrix for behavioural measurements and percentage represented by microglia population in 8 different brain structures. (Saline: n=8 males/females, n=8 dams; LPS: n=8-9 males/females, n=8-9 dams; LPS & IL-1ra: n=8 males/females, n=8 dams). (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ).

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## **General discussion and conclusion**

The work presented in this thesis focused on the study of the contribution of microglia to the development and progression of brain disorders. These studies were based on the long-held belief that inflammation is a significant contributor to triggering and the progression of these debilitating chronic diseases. The association between mental health and inflammatory processes whilst propagated over a number of decades has not been matched with successful development of therapeutic targets. The most notable example of this is the use of non-steroidal anti-inflammatory drugs (NSAIDs) for the treatment of Alzheimer's disease (AD) or mood disorders. Numerous trials principally using cyclooxygenase (COX)-2 inhibitors have been generally contradictory and to large extent failed to meet expectations even though more recent analysis of data gathered from longer term trials suggest positive outcomes (Vlad et al. 2008). The major issue with this type of therapeutic approach stemmed from the fact that it targeted the end product of the inflammation pathway, more often than not in patients with full pathology. In other words, too late. Neurodegenerative disorders such as AD are associated with aging and are principally viewed as a result of general and harmful "wear and tear" process in the brain. Younger individuals possess an inherent homeostasis restoration process that depends heavily on a healthy and fully functional immune system (Ziv et al. 2006) both in the periphery and in the brain where microglia are the main effector cells for this process. During aging this function of microglia is severely compromised with the cells gradually losing their phagocytic capacity and thus their ability to clear cell debris, misfolded proteins and toxic substances leading to accumulation of this harmful material and subsequent low grade chronic inflammation (Deczkowska and Schwartz 2018) and eventually to pathology. This example provides evidence of the importance of having fully functional and healthy microglia and although this progressive downgrading in their function is a prime illustration of an aging driven change, there are other scenarios where disrupting microglia at any stage of the life span, including early development can result in dire circumstances. In the current thesis, we performed studies to investigate this aspect of microglial involvement in the developmental of mental disorders.

In the first study (Chapter 2) we chose to perform detailed investigations based largely on previous findings from our laboratories showing that systemic inflammation with a single dose of LPS in mice triggered despair-like behaviour associated with depression involved microglial activation (Aguliar-Valles et al. 2013). The objective of the follow up study presented here was to investigate the nature of microglial involvement in this process. By selectively depleting phagocytic (M2 phenotype) microglia we hoped to show a significant change in the inflammatory response resulting in the behavioural anomalies previously documented by our laboratories and others (Dantzer et al. 2008; O'Connor, Lawson, et al. 2009). Our results confirmed these findings and demonstrated a clear link between the depression-like behaviour and increased IDO expression in the brain. This rate limiting enzyme has previously been shown to be triggered by systemic LPS (O'Connor, Lawson, et al. 2009) and correlated with behavioural changes. Importantly, we showed for the first time that LPS-induced IDO expression, as well as the behavioural alterations, were almost totally abolished in mice where the phagocytic microglia were depleted. It remains unclear however what the nature of the relationship between microglia and IDO is. There is evidence to show that these cells express IDO and that release of this enzyme can be triggered by interferon- $\gamma$  (Kwidzinski and Bechmann 2007; O'Connor, Andre, et al. 2009; Wang et al. 2009; Yadav et al. 2007). The production of this cytokine itself is known to be stimulated by LPS, interestingly through an IL-1 mediated process (Le et al. 1986). Indeed, in preliminary studies using the IL-1 receptor antagonist (IL-1ra) we demonstrated a significant inhibition of the LPS induced expression of IDO in the brain (see [Figure 1](#)). Clearly, more focused investigations are required to further delineate the exact nature of these interactions, in the meantime however, our data evidently points to a mechanistic link between systemic inflammation, activation of microglia, and release of inflammatory mediators ultimately leading to the triggering of the tryptophan pathway and subsequent depression-like behaviour.

The studies implemented as part of Chapter 2 were designed to address a specific mechanistic pathway leading to abnormal (depression-like) behaviours in adult mice. The obvious limitation of this type of approach is that it only provides insight on a short-term event to

identify the steps involved. Depression and other psychiatric disorders are however chronic diseases that develop over an extended period of time in genetically susceptible individuals. The genesis of these disorders, even in cases with a susceptible genetic background, is a complex multi factorial process that is largely been recognized to be markedly influenced by the individual's immediate environment. Of the environmental factors implicated, *in utero* events during gestation have received a great deal of attention, not least because of the vulnerability of the developing fetus to the environmental challenges that the mother maybe exposed to. A casing example of this is exposure to the influenza virus, which epidemiological studies [see (Brown and Derkits 2010) for review] have linked to increased incidence of mental disorders such as SCZ in the offspring of affected mothers. Although numerous animal models have been developed to investigate the mechanisms involved including, stress, diet, pollution and others (Boksa Luheshi, 2003; Boksa, 2004), the use of immunogenic inflammatory stimuli has been the most common approach in rodents. Of those live viruses and more commonly now a viral mimic, Poly(I:C) have been the tools of choice. In the majority of our studies, including those presented in the current thesis, however, we have opted for the use of LPS for a number of reasons. Firstly we determined that it is overall a more potent immunogen when compared to poly(I:C), and we found that the responses that it triggered (mostly cytokine induction) were generally more consistent and reliable (Fortier, Luheshi, and Boksa 2007). The majority of our MIA type studies using LPS involved the administration of the immunogen at mid- to late-gestation (Ashdown et al. 2006; Fernández de Cossío et al. 2017; Fortier et al. 2004; Labrousse et al. 2018). The difference in the current thesis is that the LPS was injected at a relatively early time point in order to address the hypothesis posed. Given that this was the first time that such an early time point was used we performed studies to characterize the type of response triggered and at the same time test our hypothesis using a proof of principle approach. The outcome from this investigation (as presented in Chapter 3) confirmed the potency of the challenge as indicated by the effects on microglia population during development and behavioural alterations in the offspring. This was an important step to confirm not only the validity of the immunogen but that it triggers the type of responses that we were interested in investigating. This was also necessary given that the use of LPS is far less common in this type of model than Poly(I:C) and we were

amongst the first to opt for using it as the immunogen of choice (Ashdown et al. 2006; Fortier et al. 2004). In recent years, LPS has become more widely used in this type of research [see (Lombardo et al. 2018; Oskvig et al. 2012; Simões et al. 2018; Squarzoni et al. 2014) recent examples] and despite the fact that it is, on balance, less clinically relevant than the use of viruses or viral mimics, it still triggers the same inflammatory pathways making it a very important tool in this type of research.

Historically, whether using LPS or any other environmental challenge in the MIA model, by far the majority of the studies (including most of ours) performed on the offspring of the treated mothers were restricted to the male progeny. This was driven to a large extent by the fact that conducting experiments in females was deemed to be generally more complicated as menstrual cycles had to be considered and in some cases affected the outcome of the experiments. In recent years however, given the reported and very significant differences in physiological responses reported between male and female subjects this issue has now gained prominence. Interestingly these sex differences have been reported in relation to microglia and how they develop and function (Hanamsagar et al. 2017; Hanamsagar and Bilbo 2016; Lenz et al. 2013; Schwarz and Bilbo 2012). Most relevant to the studies in the current thesis is that microglial pruning of neurons was recently reported to be sex dependent (Weinhard et al. 2018). In a recent study from our laboratory (Fernández de Cossío et al. 2017), using the prenatal infection model we demonstrated clear differences in both the molecular and behavioural changes of the offspring along sex lines with changes in the male being more prominent. These results were confirmed in the studies outlined in Chapter 3 and were extended in the final chapter (Chapter 4) investigating the underlying inflammatory mechanisms modulating the changes in microglial development and behaviour of the offspring.

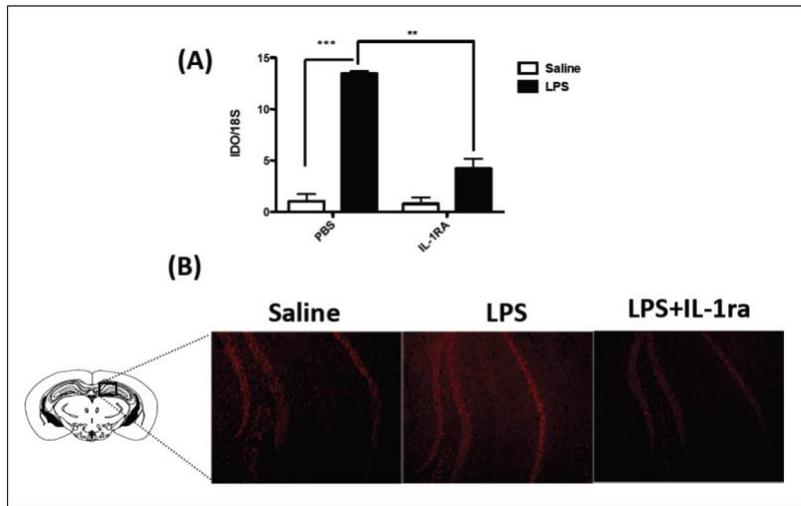
Previous studies investigating the nature of the inflammatory mediator involved in regulating the changes and subsequent development for the fetal brain identified IL-6 as the most likely culprit (Smith et al. 2007; Wu et al. 2017). These studies utilized Poly(I:C) in the presence or absence of a neutralizing antiserum for IL-6, or transgenic approaches in mice in which the IL-6 receptor was specifically deleted in placental trophoblasts (Wu et al. 2017). These

studies provided convincing evidence implicating this cytokine in relaying the inflammatory signal to the fetal brain, however, no evidence was provided as to how IL-6 does this. In addition, it would be difficult to argue that this mediator is acting through alteration of microglial development or function, since to the best of our knowledge, these cells do not express the IL-6 receptor. One possibility however is that IL-6 triggers a secondary inflammatory mediator, for example prostaglandin (PG)E<sub>2</sub>. Microglia express the PG EP<sub>2</sub> receptors which have been shown to mediate microglial activation (Quan, Jiang, and Dingledine 2013) and we have previously shown that IL-6 induces PGE<sub>2</sub> production in the brain (Rummel et al. 2008). Interestingly, PGs have been directly implicated in the developmental masculinization of sex behaviour (Amateau and McCarthy 2004). These observations add support to the notion that IL-6 is an important mediator of the inflammation-induced changes observed in our model and those of others. In addition, the results obtained in the final chapter of this thesis (Chapter 4) confirm that the levels of this cytokine do increase significantly in the multiple compartments measured, including the placenta and fetal brain. However, our results also show that IL-6 is not the main driver of these changes and most likely is acting to propagate the response triggered by the main pro-inflammatory cytokine involved, IL-1 $\beta$ . This conclusion is based on our previous findings that IL-6 is an important downstream mediator of IL-1 activity *in vivo* following an inflammatory response (Cartmell et al. 2000; Laye et al. 2000; Luheshi et al. 1996; Luheshi et al. 1997) and that IL-1 can directly modulate microglial activity (Parker et al. 2002; Pinteaux et al. 2002). In addition to these observations, the results presented in the current thesis provide perhaps the strongest evidence of the critical contribution of IL-1 in the context of the MIA model. These data show that a single dose of the receptor antagonist IL-1ra, at an early development time point injected in mice exposed to LPS offers protection against the developmental changes in microglia at the embryonic stage, noted after exposure to the immunogen and more importantly, the subsequent behavioural anomalies seen in the offspring. The potency of this inhibitor in preventing these inflammation-induced changes bodes well for its potential as a therapeutic agent that can be administered to ‘at risk’ pregnant women during early stages of gestation. Indeed, this antagonist (Anakinra) is currently being used clinically for the

treatment of rheumatoid arthritis and has been shown to be safe and effective (Cohen et al. 2002; Mertens and Singh 2009).

The other important result from the studies presented in Chapter 4 is that we confirmed the clear sexually dimorphic outcomes in the progeny of the LPS treated mothers compared to controls. This is a powerful observation that adds further justification to investigating physiological responses along sex lines, especially in a developmental context. Evidence exists that this divergence along sex lines begins at the embryonic level and specifically implicate the placenta (Bale 2016; Bronson and Bale 2016; Kalisch-Smith et al. 2017; Nugent and Bale 2015; Wu et al. 2017). Other than the role of IL-6 eluded to above and its induction of secondary inflammatory mediators (PG) other mechanisms have been proposed. A very recent study (Sept. 2018) for example demonstrated that a mutation of the *Chd8* gene, a strong ASD risk gene, underlies the sexually dimorphic nature of ASD related behaviour in mice (Jung et al. 2018). This gene is highly penetrant for ASD (Barnard, Pomaville, and O’Roak 2015; Bernier et al. 2014) and is a key master regulator of important neurodevelopmental processes such as chromatin remodeling of hundreds of genes implicated in transcription, cell division, proteolysis [all enriched during embryonic development (Barnard, Pomaville, and O’Roak 2015)], DNA integrity and signal transduction (Pramparo et al. 2015). Interestingly transcriptional and translational programs that are downstream targets of *Chd8* were shown to be significantly affected by MIA in mice treated with LPS (Lombardo et al. 2018), although interactions with maternal or fetal cytokines are not known.

In conclusion, our results clearly implicate a role for microglia in the development of aberrant behaviours associated with psychiatric brain disorders in humans. We believe that our results add further support to the potential vulnerability of the developing fetus to environmental challenges that can impact the mothers. The list of these environmental risk factors is growing and other than maternal infection/inflammation it includes stress, diet, smoking, alcohol consumption and even pollution, all features of everyday life and a sign of the potential impact that the environment and current life style have on the development of psychiatric disorders.

**Figure 1.**

**Figure 1.** (A) LPS induced a significant increase in IDO mRNA expression in the hippocampus of adult mice that was almost totally abolished in the presence of IL-1 antagonist IL-1ra. (B) Expression of IDO protein was similarly induced in the CA1 region of the hippocampus by LPS and significantly inhibited with IL-1ra.

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