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1 **Controversies and prospects about microglia in maternal immune activation**  
2 **models for neurodevelopmental disorders.**

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1 **Abstract**

2 Activation of the maternal immune system during pregnancy is a well-established risk factor for  
3 neuropsychiatric disease in the offspring, yet, the underlying mechanisms leading to altered brain  
4 function remain largely undefined. Microglia, the resident immune cells of the brain, are key to  
5 adequate development of the central nervous system (CNS), and are prime candidates to mediate  
6 maternal immune activation (MIA)-induced brain abnormalities. As such, the effects of MIA on the  
7 immunological phenotype of microglia has been widely investigated. However, contradicting results  
8 due to differences in read-out and methodological approaches impede final conclusions on MIA-  
9 induced microglial alterations. The aim of this review is to critically discuss the evidence for an  
10 activated microglial phenotype upon MIA.

11 **Keywords: Maternal immune activation, Microglia, Neurodevelopmental disorders, Poly(I:C), LPS**

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## 1 **1. Introduction**

2 Maternal immune activation during pregnancy has been recognized to increase the risk for  
3 neurodevelopmental psychiatric disorders such as schizophrenia, Autism Spectrum Disorders (ASD)  
4 and bipolar disorder. Numerous epidemiological studies identified a number of inflammation-inducing  
5 agents to be associated with psychiatric illnesses including prenatal exposure to viruses such as rubella,  
6 herpes simplex, measles and polio (Torrey *et al.* 1988, Suvisaari *et al.* 1999, Brown *et al.* 2001, Buka *et*  
7 *al.* 2001), various bacterial pathogens (Sorensen *et al.* 2009), and protozoan parasite such as  
8 *Taxoplasma gondii* (Mortensen *et al.* 2007). Animal models of maternal immune activation (MIA) have  
9 further established causality by showing that immune activation during pregnancy can perturb  
10 processes of early neurodevelopment in the offspring and change developmental trajectories. These  
11 MIA models precipitate into long-term behavioral and neuronal deficits relevant for psychiatric  
12 illnesses such as schizophrenia, ASD and related disorders (as reviewed in a.o. (Boksa 2010, Meyer  
13 2014, Solek *et al.* 2017)).

14 In the MIA model, the maternal immune system can be stimulated by a variety of immune-activating  
15 agents including virus ((Fatemi *et al.* 1998) and others), lipopolysaccharide (LPS) ((Borrell *et al.* 2002)  
16 and others) and polyriboinosinic-polyribocytidilic acid (Poly(I:C)) ((Meyer *et al.* 2005) and others)  
17 resulting in viral, bacterial-like and viral-like immune responses respectively. Intriguingly, the structural  
18 and functional abnormalities in offspring exposed to MIA seem not to be dependent on the immune-  
19 activating agents per se but critically depend on the general maternal and fetal immune response  
20 (Smith *et al.* 2007, Choi *et al.* 2016). Specific cytokines including interleukin (IL) 6 and IL17a have been  
21 identified as key players in mediating the disruption of neurodevelopmental processes in the MIA  
22 models (Smith *et al.* 2007, Choi *et al.* 2016). Moreover, the importance of the maternal gut microbiota  
23 in the response to MIA has recently been eluded by Kim *et al.* 2017, showing that treatment with the  
24 broad spectrum antibiotic vancomycin prior to MIA was able to prevent the emergence of behavioral  
25 abnormalities in the offspring (Kim *et al.* 2017). Despite this, however, the precise mechanisms through

1 which prenatal immune activation perturbs early brain development and leads to behavioral and  
2 neuronal deficits remain largely unknown. In recent years, there has been an increasing interest in the  
3 functional contribution of the neuro-immune system, and in particular aberrant microglia function, in  
4 the pathophysiological processes underlying neurodevelopmental disorders such as schizophrenia and  
5 ASD.

6 Microglia are the resident immune cells of the central nervous system (CNS). They are derived from  
7 primitive yolk-sac macrophages that invade the brain during early development (Ginhoux *et al.* 2010).  
8 In the adult brain, microglia actively scan the CNS parenchyma for potential changes in brain  
9 homeostasis or harmful insults by protruding and retracting their processes (Nimmerjahn *et al.* 2005).  
10 Once microglia sense changes in CNS integrity, they react accordingly. The classical response involves  
11 a process that is often referred to as “microglia activation” and includes changes in the expression  
12 profile of distinct markers of microglia, increased production and secretion of pro- and anti-  
13 inflammatory cytokines and chemokines, and a shift in the morphological appearance from ramified  
14 to a more amoeboid morphology (Ransohoff and Cardona 2010). This classical immune-related  
15 activation refers to microglia responses towards harmful stimuli such as damage or invading  
16 pathogens. Over the past decade, however, the functional role of microglia has been extended to non-  
17 immunological functions including the regulation of neurogenesis, myelination and synaptic  
18 remodeling (Bilimoria and Stevens 2015, Salter and Stevens 2017). As a consequence, the sustained  
19 activity of microglial protrusions might also reflect a continuous contribution in the ongoing  
20 remodeling of the brain instead of simply scanning the brain (Mosser *et al.* 2017). Microglia have thus  
21 been attributed a key role in neurodevelopmental processes, including the establishment of proper  
22 neuronal connectivity and brain homeostasis (as extensively reviewed in (Arnold and Betsholtz 2013,  
23 Bilimoria and Stevens 2015, Wu *et al.* 2015, Frost and Schafer 2016, Reemst *et al.* 2016, Mosser *et al.*  
24 2017, Paolicelli and Ferretti 2017)). Given these neuro-modulatory functions, abnormal microglia  
25 activity may thus alter neuronal development and thereby contribute to the behavioral and neuronal  
26 deficits observed in neurodevelopmental disorders.

1 Consequently, it has been proposed that prenatal immune activation could act directly on microglia  
2 and thereby alter their function in the developing brain, resulting in changing the developmental  
3 trajectories and ultimately leading to the behavioral and neuronal deficits detected in MIA models  
4 (Knuesel *et al.* 2014, Paolicelli and Ferretti 2017). In support of this hypothesis is the identification of  
5 signs of central inflammatory processes and microglia anomalies in post-mortem brains of a subset of  
6 schizophrenic and ASD patients (Vargas *et al.* 2005, Morgan *et al.* 2010, Trepanier *et al.* 2016, van  
7 Kesteren *et al.* 2017). Besides this, there have been numerous studies that investigated microglia  
8 phenotypes in the MIA model in order to associate microglia activity with brain and behavioral  
9 abnormalities (Arsenault *et al.* 2014, Bilbo *et al.* 2017, Paolicelli and Ferretti 2017). However, the  
10 reports are inconsistent with some detecting microglia with an altered phenotype (e.g. (Esslinger *et al.*  
11 2016, Gumusoglu *et al.* 2017)) whereas others detect no changes in microglia biology in offspring  
12 prenatally exposed to MIA (e.g. (Giovanoli *et al.* 2015, Smolders *et al.* 2015)). It is one of the main aims  
13 of this review to thoroughly discuss the controversies in the field and critically revise possible factors  
14 that could contribute to the controversial findings regarding microglia alterations in the MIA model.  
15 Among these factors are those associated to the commonly used microglial activation read-outs (such  
16 as the age, sex and brain region analyzed) as well as MIA-model associated factors (such as the immune  
17 stimulant used and the age of MIA induction). We aim to conclude with suggestions/guidelines to  
18 improve the consistency in the field of MIA-microglia research.

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## 20 **2. Methods**

21 The publications discussed in paragraph 3 were retrieved by searching the Title and/or Abstract field  
22 in pubmed using the following search terms: ‘maternal immune activation’, ‘prenatal immune  
23 activation’, ‘maternal immune stimulation’, ‘prenatal immune stimulation’, ‘maternal immune  
24 challenge’, ‘prenatal immune challenge’, ‘prenatal infection’, ‘prenatal viral infection’, ‘prenatal  
25 bacterial infection’, ‘maternal viral infection’, ‘maternal bacterial infection’, ‘maternal infection’ or

1 'maternal inflammation'. Every publication found by these search terms was opened and inspected for  
2 analysis on microglia. Whilst the MIA model has been extended to pigs (Antonson *et al.* 2017) and  
3 (non)-human primates (Careaga *et al.* 2017) we will only discuss and revise studies that systemically  
4 administered immune agents such as LPS or Poly(I:C) or specific cytokines (excluding intrauterine  
5 administration) in rodents including mice and rats. Although early postnatal injections (either through  
6 direct infection of the neonate or through infection of the lactating dam) of Poly(I:C) have been used  
7 to mimic late-gestation infection in humans (Ribeiro *et al.* 2013, Arad *et al.* 2017, Monte *et al.* 2017),  
8 we will only consider studies inducing MIA at prenatal stages in order to include the effects of the  
9 maternal immune system and contribution of the placenta.

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### 11 **3. Microglial reaction to maternal infection**

12 The findings on MIA-induced alterations of microglia activity in rodents are divergent. There are  
13 reports that describe alterations in the microglia activity state that point towards a pro-inflammatory  
14 phenotype. These include increased microglia density (here defined as amount of cells/mm<sup>2</sup>) (e.g.  
15 (Juckel *et al.* 2011, Manitz *et al.* 2013, Li *et al.* 2014, Zhu *et al.* 2014)), increased expression of microglia  
16 "activation" markers (e.g. Major histocompatibility complex II (MHCII), Cluster of differentiation (CD)  
17 68 or CD54 (Borrell *et al.* 2002, Van den Eynde *et al.* 2014, Esslinger *et al.* 2016)), and changes in the  
18 morphological appearance (e.g. altered microglial branches (Juckel *et al.* 2011, Van den Eynde *et al.*  
19 2014, Corradini *et al.* 2017)). On the other hand, there are multiple studies where no obvious changes  
20 in microglia activity were detected (e.g. (Garay *et al.* 2013, Giovanoli *et al.* 2015, Giovanoli *et al.* 2016)).  
21 Multiple reasons may underlie these contradictory results and should be considered when comparing  
22 the outcome of different studies on "microglia activation".

23 Firstly, "microglial activation" can be assessed in several ways, some of which are more sensitive than  
24 others. The most common methods used in MIA models include the estimation of the number/density

1 of microglia by means of stereology (e.g. (Mattei *et al.* 2014, Giovanoli *et al.* 2015, Hadar *et al.* 2016)),  
2 and the morphological appearance of microglia (e.g. (Giovanoli *et al.* 2015, Hsueh *et al.* 2017, Notter  
3 *et al.* 2017)). Another common approach to measure microglia activity is the assessment of the  
4 expression – usually by means of immunohistochemistry or biochemistry – of microglia activation  
5 markers (such as CD68 (Esslinger *et al.* 2016, Manitz *et al.* 2016, Notter *et al.* 2017) and Translocator  
6 protein (TSPO) (Mattei *et al.* 2017, Notter *et al.* 2017). To identify or isolate microglia from the brain,  
7 samples obtained from non-transgenic animals are stained with markers that are highly expressed by  
8 microglia (such as ionized calcium-binding adapter molecule 1 (Iba1), CD11b or CD68) or samples are  
9 obtained from transgenic animals where a reporter gene is expressed under the CX3C chemokine  
10 receptor 1 (CX3CR1) promotor. Although these molecular markers for microglia are often considered  
11 to be microglia-specific, they are also expressed by other members of the mononuclear phagocyte  
12 system such as monocytes and macrophages. Therefore, many groups have recently searched for a  
13 molecular marker that allows to distinguish between microglia and other immune cells in the brain. As  
14 such, the expression of the transcriptional repressor Spalt Like Transcription Factor 1 (Sall1) was  
15 recently shown to be largely restricted to microglia in the adult brain, although some cells in the liver,  
16 kidney and heart were also found to express this marker (Buttgereit *et al.* 2016). Similarly,  
17 Transmembrane protein 119 (Tmem119) was identified as a microglia-specific marker in the human  
18 and mouse CNS (Bennett *et al.* 2016). Although these markers will be of great value to distinguish  
19 microglia in the adult brain, their use in embryonic contexts should be further investigated.

20 Besides the methodology and readouts used to assess microglia activation state, the brain region  
21 investigated and time point/developmental stage when microglia are analyzed differ among the  
22 different studies, which makes direct comparisons merely impossible. In addition to these analytical  
23 issues, there are numerous other factors to be considered including the immune stimulant used,  
24 gestational window of immune activation, frequency of immune challenges, and route of  
25 administration that could all contribute to the observed inconsistencies in microglia phenotypes in the  
26 MIA model. Moreover, factors that are not directly related to the method of MIA-induction or microglia



1 activation assessment could influence the outcome of MIA studies, including housing conditions, food  
2 pellet composition and specific mouse strain (Babri *et al.* 2014, Li *et al.* 2015, Buschert *et al.* 2016,  
3 Weiser *et al.* 2016).

4 The vast majority of studies have analyzed MIA-induced alterations of microglial activity during  
5 postnatal stages while only a few studies have focused on microglial changes during prenatal  
6 developmental stages. In the first section, we focus on findings derived from offspring in postnatal  
7 stages subdivided according to the specific read-out used to assess microglia activation  
8 (number/density, morphology, expression of microglia activity dependent genes and proteins) in order  
9 to compare different MIA-microglia studies easier. However, as will be discussed later in this review,  
10 even by dividing studies in this manner, many other variables make direct comparison merely  
11 impossible. In the subsequent section, findings obtained during prenatal development are discussed.

### 12 **3.1 Readout Postnatal Stage**

#### 13 *Microglia Number/Density*

14 Upon detection of an insult, microglia are known to migrate to the site of injury/infection and/or  
15 proliferate, a phenomenon called microgliosis (Streit *et al.* 1999). “Microglial activation” is thus often  
16 measured in relation to its number/density in specific brain regions of interest. Despite seemingly  
17 straightforward, the density of microglia can vary depending on the age and sex of the animal, as well  
18 as the brain region investigated (Schwarz *et al.* 2012) (as will be discussed in section 4). Moreover,  
19 studies differ with regard to the marker used to identify microglia (Iba1 or CD11b) and therefore, in  
20 the tables below, the specific markers are mentioned. In addition, the variety in techniques to  
21 determine microglia density (fluorescent vs chromogenic staining and software) might lead to  
22 variability in results between different studies.

23 A total of 21 studies were included in this review assessing microglia density in different brain regions  
24 of rat and mice offspring exposed to MIA (Table 1). Out of these, 19 studies measured microglia in

1 adult offspring (age > P60). A minority of these studies (only 5) reported an increased density of  
 2 microglia in several brain regions while the vast majority (14 studies) did not detect a change in  
 3 microglial density. Similar to the findings in adult brain, measurements in younger, adolescent  
 4 offspring provides more evidence for unchanged microglial densities (4 studies) in comparison to  
 5 increased density (only 2 studies).

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

9 **Table 1: Microglia number/density in postnatal MIA offspring**

<b>Microglia Number/Density</b>				
<b>Toxin/ Microglia marker/ Ref.</b>	<b>Species/ Dose/ Gestation Day</b>	<b>Age of Analysis</b>	<b>No Change</b>	<b>Increase</b>
<b>Poly(I:C)</b> Iba1 (Juckel <i>et al.</i> 2011)	BALB/c mice/ 20mg/kg (i.p.)/ GD 9	PND 30	Cx	HPC, Str
<b>Poly(I:C)</b> CD11b (Hsiao <i>et al.</i> 2012)	C57BL6 mice/ 20mg/kg (i.p.) GD 12.5	Adult	Whole brain	
<b>Poly(I:C)</b> Iba1 (Garay <i>et al.</i> 2013)	C57BL6 mice/ 20mg/kg (i.p.) GD 12.5	PND 0, PND 7, PND 14, PND 30, PND 60	FCx, CgCx, HPC	

<b>Poly(I:C)</b>	BALB/c mice/	PND 10	Str, FaCx, HPC,	
Iba1	20mg/kg (i.p.)		VCx, MCx	
(Manitz <i>et al.</i> 2013)	GD 9.5	PND 30	Str, MCx	FaCx, HPC
		PND 100	HPC, VCx, MCx	Str, FaCx
<b>Poly(I:C)</b>	C57BL6 mice/	PND 85	mPfcx, Str	
Iba1	5mg/kg (i.v.)			
(Willi <i>et al.</i> 2013)	GD 9			
<b>Poly(I:C)</b>	C57BL6 mice/	PND 2		HPC
Iba1	20mg/kg (i.p.)	PND 80	HPC	
(Li <i>et al.</i> 2014)	GD 9.5			
<b>Poly(I:C)</b>	C57BL6 mice/	PND 62		Th, Cx, HPC
Iba1	20mg/kg (i.p.)			
(Zhu <i>et al.</i> 2014)	GD 9			
<b>Poly(I:C)</b>	C57BL6 mice/	PND 28, PND	HPC	
Iba1	5mg/kg (i.v.)	140, PND 600		
(Giovanoli <i>et al.</i> 2015)	GD 17			
<b>Poly(I:C)</b>	CD-1 mice/	PND 172		
Iba1	20mg/kg (i.p.)	Standard	Cx, HPC	
(Buschert <i>et al.</i> 2016)	GD 9.5	housing		
		Enriched	Cx, HPC	
		housing		

<b>Poly(I:C)</b>	C57BL6 mice/	PND 35, PND 84	HPC
Iba1	5mg/kg (i.v.)		
(Giovanoli <i>et al.</i> 2016)	GD 9		
<b>Poly(I:C)</b>	C57BL6 mice/	PND 90	Cx
Iba1	2mg/kg (i.p.)		
(Corradini <i>et al.</i> 2017)	GD 9		
<b>Poly(I:C)</b>	C57BL6 mice/	PND 90	mPfcx
Iba1	5mg/kg (i.v.)		
(Notter <i>et al.</i> 2017)	GD 9		
<b>Poly(I:C)</b>	C57BL6 mice/	PND 80	Pfcx, Cb, HPC
Iba1	5mg/kg (i.p.)		
(Hui <i>et al.</i> 2018)	GD 9.5		
<b>Poly(I:C)</b>	Lewis rat/	PND 9	Forebrain
Iba1	200 µg/kg		White matter
(Girard <i>et al.</i> 2010)	(i.p.)/every 12h starting from GD 17.5 until birth		
<b>Poly(I:C)</b>	Wistar rat/	PND 128	Str, Cb, HPC, Pfcx NAc
Iba1	4 mg/kg (i.v.)		
(Mattei <i>et al.</i> 2014)	GD 15.5		

<b>Poly(I:C)</b>	Wistar-Hannover	PND 90	FtCx, CC, HPC,	
CD11b/Iba1	rat/		Th, Str, Pons	
(Missault <i>et al.</i>	4 mg/kg (i.v.)			
2014)	GD 15.5			
<b>Poly(I:C)</b>	Sprague Dawley	PND 180	Th, Cx, HPC, Str,	Pons, CC
CD11b	rat/			
(Van den Eynde <i>et</i>	4 mg/kg (i.v.)			
<i>al.</i> 2014)	GD 15.5			
<b>Poly(I:C)</b>	Wistar rat/	PND 130	PfCx	NAC, HPC
Iba1	4 mg/kg (i.v.)			
(Hadar <i>et al.</i> 2016)	GD15.5			
<b>Poly(I:C)</b>	Long-Evans rat/	PND 7, PND 21,	MpCx, FaCx,	
Iba1	4 mg/kg (i.v.)	PND 35, PND 90	PaCx, Am	
(Paylor <i>et al.</i> 2016)	GD 15.5			
<b>LPS</b>	C57BL6 mice/	PND 56	Cx	
Iba1	25-50 $\mu$ /kg (i.v.)			
(Hsueh <i>et al.</i> 2017)	GD 15 - 17			
<b>LPS</b>	Sprague Dawley	PND 70	HPC	
Iba1	rat/			
(Mouihate 2016)	100 $\mu$ g/kg (i.p.)			
	GD 15.5			

1 **HPC: Hippocampus, NAc: Nucleus accumbens, Str: Striatum, Th: Thalamus, Cx: Cortex, CgCx:**  
2 **Cingulate Cortex, VCx: Visual Cortex, MCx: Motor cortex, FaCx: Frontal association Cortex, FCx:**  
3 **Frontal Cortex, FtCx: Frontotemporal Cortex, (m)Pfcx: (medial) Prefrontal Cortex, MpCx: Medial**  
4 **Prelimbic Cortex, PaCx: Primary Auditory Cortex, CC: Corpus Callosum, Cb: Cerebellum, Am:**  
5 **Amygdala**

## 6 *Microglia Morphology*

7 A second parameter often used to assess “microglia activation” is the cell’s morphology. The fact that  
8 pathological insults can cause microglia to adopt a more amoeboid shape has led to the assumption  
9 that ramified microglia represent a “resting” state while amoeboid forms were interpreted as  
10 “activated” state (Hanisch and Kettenmann 2007). Single cell transcriptome studies in microglia,  
11 however, revealed that microglia activation states cannot be simply classified into “resting” and  
12 “active” states but rather represents a continuum at which ramified microglia represent an active form  
13 as well (Crotti and Ransohoff 2016). Hence, equating microglia morphology with microglia activation  
14 could therefore result in false negative results and misleading conclusions. Indeed, changes in  
15 microglia morphology represent an adequate response towards a specific insult but should not be  
16 taken as a general sign of microglia activation (Harry and Kraft 2012, Streit *et al.* 2014). On top of that,  
17 during brain development, the morphology of microglia is changing gradually from amoeboid to  
18 ramified and the age of the animals at which microglial morphology is studied might contribute to  
19 contradictory results. Conversely, the fact that microglia are ramified does not necessarily mean they  
20 are not responsive to an inflammatory stimulus on the transcriptional level: it was shown that 2-12  
21 hours after a systemic inflammatory stimulus, transcriptional changes occurred in microglia while  
22 changes in morphology were only detected after 24-48 hours (Norden *et al.* 2016).

23 Nevertheless, multiple MIA studies have taken morphological measures to assess microglia activity  
24 (Table 2). Similar to the microglia density analyses, the assessment of microglial morphology in MIA  
25 has resulted in contradictory findings. The findings of these studies vary from decreased numbers of

1 processes (Juckel *et al.* 2011, Van den Eynde *et al.* 2014, Corradini *et al.* 2017, O'Loughlin *et al.* 2017,  
 2 Hui *et al.* 2018) – indicative of the amoeboid state – to increased ramification (Gumusoglu *et al.* 2017).  
 3 In comparison to these 6 studies, however, the 7 other studies presented in this review detected no  
 4 changes in the microglia morphology of MIA exposed adult offspring (Garay *et al.* 2013, Missault *et al.*  
 5 2014, Giovanoli *et al.* 2015, Giovanoli *et al.* 2016, Mouihate 2016, Hsueh *et al.* 2017, Notter *et al.* 2017).

6 **Table 2: Microglia morphology in postnatal MIA offspring**

<b>Microglia Morphology</b>				
<b>Toxin/ Microglia marker/ Ref.</b>	<b>Species/ Dose/ Gestation Day</b>	<b>Age of Analysis</b>	<b>Regions analyzed</b>	<b>Findings</b>
<b>Poly(I:C)</b> Iba1 (Juckel <i>et al.</i> 2011)	BALB/c mice/ 20mg/kg (i.p.)/ GD 9	PND 30	HPC, Cx, Str	Decreased density and amount of branches and processes
<b>Poly(I:C)</b> Iba1 (Garay <i>et al.</i> 2013)	C57BL6 mice/ 20mg/kg (i.p.) GD 12.5	PND 0, PND 7, PND 14, PND 30, PND 60	FtCx, HPC, CgCx	No change in morphology
<b>Poly(I:C)</b> Iba1 (Giovanoli <i>et al.</i> 2015)	C57BL6 mice/ 5mg/kg (i.v.) GD 17.5	PND 28, PND 140, PND 600	HPC	No change in morphology (cell soma area and primary or secondary branches)

<b>Poly(I:C)</b>	C57BL6 mice/				
Iba1	5mg/kg (i.v.)		P35, P84	HPC	No change in morphology
(Giovanoli <i>et al.</i> 2016)	GD 9.5				(cell soma area and primary or secondary branches)
<b>Poly(I:C)</b>	C57BL6 mice/				Decreased microglial
Iba1	2mg/kg (i.p.)		PND 90	Cx	branches per cell
(Corradini <i>et al.</i> 2017)	GD 9				No change in length of microglia branches
<b>Poly(I:C)</b>	C57BL6 mice/				No change in cell soma area
Iba1	5mg/kg (i.v.)		PND 90	mPfcx	or primary branches of Iba1+-positive cells
(Notter <i>et al.</i> 2017)	GD 9.5				
<b>Poly(I:C)</b>	C57BL6 mice/		PND 80		Reduced arborization and
Iba1	5mg/kg (i.p.)			HPC	increased process area
(Hui <i>et al.</i> 2018)	GD 9.5				
<b>Poly(I:C)</b>	Wistar-Hannover				
CD11b/Iba1	rat/		PND 90	FtCx, HPC, Str,	No change in morphology
(Missault <i>et al.</i> 2014)	4 mg/kg (i.v.)			CC, Pons, Th	
	GD 15.5				
<b>Poly(I:C)</b>	Sprague Dawley				
CD11b	rat/		PND 180	Cx, HPC, Str, CC,	Reduced arborization
(Van den Eynde <i>et al.</i> 2014)	4 mg/kg (i.v.)			Pons, Th	
	GD 15.5				



<b>LPS</b>	C57BL6 mice/				
Iba1	25-50µg/kg (i.v.)	PND 56	Cx		No change in morphology
(Hsueh <i>et al.</i> 2017)	GD 15 - 17				
<b>LPS</b>	C57BL6 mice/				Increased percentage of
Iba1	50µg/kg (i.p.)	PND 7,			amoeboid microglia and
(O'Loughlin <i>et al.</i> 2017)	GD 12	PND 14,	Am		decreased percentage of
		PND 40			ramified microglia at PND 7 and PND 40
<b>LPS</b>	Sprague Dawley				
Iba1	rat/				
(Mouihate 2016)	100 µg/kg (i.p.)	PND 70	HPC		No change in morphology
	GD 15.5				
<b>IL6</b>	CD-1 Mice/ 100ng				
Iba1	(i.p.)/GD 12.5-birth				
(Gumusoglu <i>et al.</i> 2017)	3x/day	PND 84	Cx		More highly ramified microglia

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2 **HPC: Hippocampus, Str: Striatum, Th: Thalamus, Cx: Cortex, CgCx: Cingulate Cortex, FtCx:**

3 **Frontotemporal Cortex, (m)Pfcx: (medial) Prefrontal Cortex, CC: Corpus Callosum, Am: Amygdala**

4

5 *Expression of microglia activity dependent genes and proteins*

6 The analysis of a single aspect in the continuum of microglia activation states is insufficient to capture

7 subtle changes in microglia reactivity, especially in the MIA model, where subtle changes in microglia

1 activity have been detected using highly sensitive approaches including microglia-specific mRNA  
2 sequencing (Matcovitch-Natan *et al.* 2016). In order to capture subtle changes, one has to either apply  
3 microglia-specific protein or mRNA expression approaches or apply several complementary  
4 approaches. A widely used approach is to complement microglia density and morphological analyses  
5 with assessing the expression of microglial surface markers as well as cytokine and chemokine levels.  
6 Amongst the most widely used microglia markers are: CD68 (a lysosomal-associated membrane  
7 glycoprotein known to be upregulated in pro-inflammatory activity states of microglia), CD11b (subunit  
8 of the complement receptor 3 complex), MHCII, CX3CR1, CD200R (cell surface receptor of CD200,  
9 transmits inhibitory signals to microglia), and Iba1 as well as various different pro- (IL1 $\beta$ , IL6, Tumor  
10 necrosis factor  $\alpha$  (TNF $\alpha$ )) and anti-inflammatory (IL-5, IL10) cytokine and chemokine levels.

11 Again, the findings in the expression pattern of microglia markers in MIA models are diverse (Table 3).  
12 In addition, not all studies have implemented all complementary methods described before which  
13 precludes the determination of a microglia phenotype to some extent. For example, one study  
14 reported a decreased immunoreactivity of Iba1 in the dentate gyrus of MIA offspring with no changes  
15 in cell density (Mattei *et al.* 2014). In contrast, another study found an increased immunoreactivity of  
16 Iba1 in the dentate gyrus upon MIA (Mattei *et al.* 2017). In the latter study, the interpretation of what  
17 Iba1 expression changes signify when presented isolated from other microglia analyses can be difficult.  
18 For example, an observed increase in Iba1 could signify increased expression of Iba1 by microglia,  
19 increased density of microglia, increased microglia soma size (indicative for pro-inflammatory state) or  
20 an increase in ramification. Hence, the combination of other measures such as Iba1 expression with  
21 microglia density (Mattei *et al.* 2014) can aid the interpretation of the obtained results. In this example,  
22 decreased Iba1 expression together with no changes in density could be an indicator for either  
23 decreased expression of Iba1, or altered morphology with less ramification and smaller soma size.  
24 Findings from other microglia marker expression studies have yielded similarly contradicting results.  
25 In summary, while 8 out of 13 studies found no change in the expression of CD68, CD11, or Iba1, 7

1 studies did find changes in the expression (either increased or decreased) of these same markers and  
 2 others (MHCII, CD124, CX3CR1, etc.)(Table 3).

3 Two recent studies measured changes in the expression of the mitochondrial TSPO in the brains of MIA  
 4 offspring, similar to *in vivo* PET imaging studies in humans (Mattei *et al.* 2017, Notter *et al.* 2017). Both  
 5 studies find opposite results, with increased TSPO in one study and decreased TSPO in the other. The  
 6 reason for this discrepancy could be the difference in brain region (prefrontal cortex vs hippocampus)  
 7 or the difference in the MIA protocol (i.v. vs i.p. administration of Poly(I:C) at GD9 or GD17). As  
 8 discussed by the authors and recently reviewed in Notter *et al.* 2018, the use of TSPO to assess the  
 9 activation status of microglia is debatable as it is also expressed in other CNS cell types, including  
 10 astrocytes and endothelial cells (Notter *et al.* 2017, Tronel *et al.* 2017, Notter *et al.* 2018). The increase  
 11 in TSPO could thus also signify processes other than inflammation, such as oxidative stress or cell  
 12 activity per se.

13 **Table 3: Microglia surface marker expression in postnatal MIA offspring**

<b>Microglia Surface Marker Expression</b>				
<b>Toxin/ Ref.</b>	<b>Species/ Dose/ Gestation Day</b>	<b>Age of Analysis</b>	<b>Regions analyzed</b>	<b>Findings</b>
<b>Poly(I:C)</b> (Krstic <i>et al.</i> 2012)	C57Bl6 mice/ 5mg/kg (i.v.)/ GD 17.5	PND 540	HPC	No change in CD68 immunoreactivity
<b>Poly(I:C)</b> (Pineda <i>et al.</i> 2013)	C57Bl6 mice/ 2.5mg/kg (i.p.)/ GD 12 - 16	PND 40	HPC	No change in CD11b expression

<b>Poly(I:C)</b>	C57Bl6 mice/			
(Willi <i>et al.</i> 2013)	5mg/kg (i.v.)/ GD 9.5	PND 85	mPFC, Str	No change in amount of cells expressing CD68
<b>Poly(I:C)</b>	BALB/c mice/			
(Esslinger <i>et al.</i> 2016)	20mg/kg (i.p.)/ GD 9.5	PND 30		No change in amount of cells expressing CD40, CD80, CD68, MHCII, CD200R, CD11c, CCR7; Decreased amount of cells expressing CD124, CD206, CX3CR1; Increased amount of cells expressing CD54, CCR2
			Whole brain	No change in amount of cells expressing CD40, CD54, CD80, CD86, MHCII, CD124, CD206, CD200R, CD11c, CCR7, CCR2; Decreased amount of cells expressing CX3CR1
<b>Poly(I:C)</b>	C57BL6 mice/			
(Giovanoli <i>et al.</i> 2016)	5mg/kg (i.v.)/ GD 9.5	PND 35, PND 84	HPC	No change in amount of cells expressing CD68
<b>Poly(I:C)</b>	BALB/c mice/	PND 10, PND 30,	Whole brain	Decreased CD11b and CD45 No change in Iba1

(Manitz <i>et al.</i> 2016)	20mg/kg (i.9.)/ GD 9	PND 100		
<b>Poly(I:C)</b>	C57BL6 mice/			Increased Iba1 reactivity
(Mattei <i>et al.</i> 2017)	5mg/kg (i.p.)/ GD 15.5	PND 120	HPC	Increased TSPO binding potential
<b>Poly(I:C)</b>	C57BL6 mice/			No change in amount of cells expressing CD11b
(Corradini <i>et al.</i> 2017)	2mg/kg (i.p.)/ GD 9	PND 90	Cx	
<b>Poly(I:C)</b>	C57BL6 mice/			No change in CD68 optical density
(Notter <i>et al.</i> 2017)	5mg/kg (i.v.)/ GD 9	PND 90	mPFC, HPC	Decreased TSPO density
<b>Poly(I:C)</b>	Wistar Rat/			
(Mattei <i>et al.</i> 2014)	4mg/kg (i.v.)/ GD 15.5	PND 128	HPC, Cb	Decreased Iba1 reactivity
<b>Poly(I:C)</b>	Sprague Dawley			
(Van den Eynde <i>et al.</i> 2014)	Rat/ 4mg/kg (i.v.)/ GD 15.5	PND 180	Cx, HPC, Str, CC, Pons, Th	Increased CD11b or CD68 reactivity
<b>LPS</b>	C57BL6 mice/			
(Hsueh <i>et al.</i> 2017)	25-50µg/kg (i.v.) GD 15 - 17	PND 56	Cx	No change in Iba1 optical density
<b>LPS</b>	Wister rat/			
(Borrell <i>et al.</i> 2002)	1mg/kg (s.c.)/	P100	Internal capsule	Increased MHCII immunoreactivity

1

2 **HPC: Hippocampus, Str: Striatum, Th: Thalamus, Cx: Cortex, (m)PfCx: (medial) Prefrontal Cortex, CC:**  
3 **Corpus Callosum, Cb: Cerebellum**

4 Besides microglia surface markers, a number of studies have assessed the expression of pro- and anti-  
5 inflammatory cytokines in MIA offspring (Table 4). Although not specific for microglia, several studies  
6 determined cytokine levels in brain homogenates in an attempt to give an idea about the inflammatory  
7 status of the brain. However, cytokines are not only produced by microglia, but also by other  
8 parenchymal cell types (such as astrocytes and neurons) and even by cells in the meningeal space.  
9 Moreover, many cytokines (such as IL1 $\beta$ , IL6 and TNF $\alpha$ ) not only play a role in inflammation but also in  
10 modulation of synaptic transmission and plasticity (as reviewed in (Vezzani and Viviani 2015)). Cytokine  
11 levels can be measured either on the mRNA or on the protein level but differences in transcriptional  
12 and translational dynamics might create additional variation between studies. In total, 8 studies  
13 determined cytokine levels in brain homogenates and findings are divergent. Yet, this might also be  
14 explained by dynamic changes in several brain cytokines in a region- and age-specific manner of early  
15 postnatal to adult MIA offspring (PND 0 – 60) (Garay *et al.* 2013). To our knowledge, only two studies  
16 measured cytokine levels (on the mRNA level) in isolated microglia from adult MIA offspring. Strikingly,  
17 although the protocols used to induce MIA and the age and brain region (hippocampus) analyzed are  
18 the same, Mattei *et al.* 2014 showed increased levels of IL1 $\beta$  mRNA while Hadar *et al.* 2016 found no  
19 change in IL1 $\beta$  mRNA levels (Mattei *et al.* 2014, Hadar *et al.* 2016).

20 **Table 4: Microglia cytokine expression in postnatal MIA offspring**

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**Cytokine mRNA/protein Expression**

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Toxin/ Ref.	Species/ Dose/ Gestation Day	Age of Analysis	Regions analyzed	No change	Increase
<b>Poly(I:C)</b> (Mattei <i>et al.</i> 2014)	Wistar Rat/ 4mg/kg (i.v.)/ GD 15.5	PND 128	HPC, Cb	TNF $\alpha$ TNF $\alpha$ , IL1 $\beta$	IL1 $\beta$
<b>Poly(I:C)</b> (Hadar <i>et al.</i> 2016)	Wistar Rat/ 4mg/kg (i.v.)/ GD 15.5	PND 130	HPC, mPFC	TNF $\alpha$ , IL1 $\beta$ , IL6	
<b>Poly(I:C)</b> (Krstic <i>et al.</i> 2012)	C57Bl6 mice/ 5mg/kg (i.v.)/ GD 17.5	PND 540	Cx, HPC	IL1 $\alpha$ , IL6, TNF $\alpha$	IL1 $\alpha$ , IL6
<b>Poly(I:C)</b> (Garay <i>et al.</i> 2013)	C57Bl6 mice/ 2mg/kg (i.p.)/ GD 12.5	PND 0, PND 7, PND 14, PND 30, PND 60	HPC, FCx, CgCx	TNF $\alpha$	IL1 $\alpha$ , IL1 $\beta$ , IL6, IL10, IFN $\gamma$ , IL2, IL3, IL4, IL5, IL9, IL12(p40) IL12(p70), IL13, IL17, G-CSF, GM-CSF, eotaxin, KC, MCP-1, MIP1 $\alpha$ , MIP1 $\beta$ , RANTES (increased/decreased)
<b>Poly(I:C)</b> (Giovannoli <i>et al.</i> 2015)	C57Bl6 mice/ 5mg/kg (i.v.)/ GD 17.5	PND 28, PND	HPC	IL1 $\beta$ , IL4, IL6, TNF $\alpha$	

		140,			
		PND 600			
<b>Poly(I:C)</b>	C57BL6 mice/	PND 40,		IL4, IL6, TNF $\alpha$ ,	
(Giovanoli <i>et al.</i>	5mg/kg (i.v.)		HPC	IL1 $\beta$	
2016)	GD 9	PND 90		IL4, IL6, TNF $\alpha$	IL1 $\beta$
<b>Poly(I:C)</b>	C57BL6 mice/	PND 90	mPFCx		
(Notter <i>et al.</i>	5mg/kg (i.v.)			IL1 $\beta$ , IL5, IL6	IL10, TNF $\alpha$ , IFN $\gamma$
2017)	GD 9				
<b>Poly(I:C)</b>	C57BL6 mice/	PND 120	HPC		
(Mattei <i>et al.</i>	5mg/kg (i.p.)			IL1 $\beta$ , TNF $\alpha$	IL6
2017)	GD 15.5				
<b>Poly(I:C)</b>	Sprague Dawley	PND 1	Whole brain	NGF, BDNF	TNF $\alpha$ (decrease)
(Gilmore <i>et al.</i>	rat/ 10mg/kg	PND 7	FCx	NGF, BDNF,	
2005)	(i.p.) GD16			TNF $\alpha$	
<b>LPS</b>	Sprague Dawley	PND 7	FCx		TNF $\alpha$ (decrease)
(Gilmore <i>et al.</i>	rat/ 100 $\mu$ g/kg				
2005)	(i.p.) GD14 - 16				
<b>LPS</b>	C57BL6 mice/	PND 7			IL1 $\beta$
(O'Loughlin <i>et</i>	50 $\mu$ g/kg (i.p.)	PND 14	Am	IL1 $\beta$ , IL6, IL10	
<i>al.</i> 2017)	GD 12	PND 40		and/or TNF $\alpha$	



1 **HPC: Hippocampus, Cx: Cortex, CgCx: Cingulate Cortex, FCx: Frontal Cortex, (m)PFCx: (medial)**  
2 **Prefrontal Cortex, Cb: Cerebellum, Am: Amygdala. The double line separates studies performed in**  
3 **isolated microglia (above) from those in brain homogenate (below).**

4 By categorizing different studies according to their method for assessing microglia activation, we  
5 aimed to ease the comparison. However, contradictory results were found between different studies,  
6 which could, at least in part, be explained by the large variety in protocols used to induce MIA.  
7 However, even when comparing studies using comparable protocols for MIA induction, the results  
8 remain contradicting. For example, using an i.v. injection of Poly(I:C) (4mg/kg) at GD 15.5 in rats, 3  
9 studies described increased microglial densities in several brain regions (Mattei *et al.* 2014, Van den  
10 Eynde *et al.* 2014, Hadar *et al.* 2016) while the same protocol did not induce changes in microglial  
11 density in 2 other studies (Missault *et al.* 2014, Paylor *et al.* 2016). Similarly, the same protocol induced  
12 no morphological changes at PND 90 in one study (Missault *et al.* 2014), while reduced arborization  
13 was observed in PND 180 offspring in another study (Van den Eynde *et al.* 2014). Despite applying the  
14 same MIA paradigm, these studies differ with regard to the age of the offspring when microglia were  
15 assessed, pointing towards a possible involvement of natural or pathological changes in cell density  
16 along the course of development

17 Taken together, when analyzing the effect of MIA on microglia in adult MIA-offspring, considerable  
18 controversy exists when only one parameter for microglia activation, i.e. density, morphology or  
19 expression of specific markers or cytokines, is assessed, even when comparing studies using the same  
20 or highly similar MIA induction protocols. In addition, the methods to detect 'microglia activation'  
21 described above are limited in their sensitivity and detect only obvious changes in microglia activation,  
22 and should therefore ideally be combined with more sensitive approaches, allowing the definition of  
23 distinct and very refined activity states. Although we conclude that morphology as a single readout is  
24 not sufficient to detect microglial activation, it is possible that subtle morphological changes are not  
25 detected with the standard microscopic analysis and in this aspect advanced and high resolution

1 imaging such as electron microscopy might shed some more light. Moreover, using direct RNA  
2 sequencing, mRNA sequencing, gene and microRNA array, quantitative proteomic analysis and  
3 chromatin accessibility analysis, several groups have defined the unique molecular signature and  
4 'sensome' of microglia and their regulatory dynamics during brain development (Hickman *et al.* 2013,  
5 Butovsky *et al.* 2014, Matcovitch-Natan *et al.* 2016, Hanamsagar *et al.* 2017, Thion *et al.* 2018}).  
6 Interestingly, using these techniques, Mattei *et al.* 2017 and Matcovitch-Natan *et al.* 2016 showed a  
7 MIA-induced deregulation of genes involved in microglial development, inflammation and  
8 phagocytosis accompanied by increased pro-inflammatory signaling and decreased phagocytic activity  
9 in microglia of MIA offspring (Maticovitch-Natan *et al.* 2016, Mattei *et al.* 2017), arguing in favor of  
10 combining several methods to more thoroughly define the effect of MIA on microglia.

### 11 **3.2 Readout at embryonic age**

12 Activation of adult microglia due to an embryonic inflammatory insult – as described in the section  
13 above – assumes some kind of activation memory. Much more strait forward is the hypothesis that  
14 embryonic microglia are activated at the time of maternal immune activation and thereby influence  
15 early neurodevelopment. The detection of increased mRNA levels of inflammatory cytokines in the  
16 embryonic brain within a few hours upon MIA (Meyer *et al.* 2006) strengthened the hypothesis that  
17 MIA could alter microglia activation states during prenatal development. Recently, the expression of  
18 sensome genes was shown already in the progenitor phase of microglia (yolk sac macrophages –  
19 GD10.5) (Thion *et al.* 2018), lending even more support to the notion that microglia are already able  
20 to respond to (pathological) changes in the environment in early stages of development. In order to  
21 test this, the microglia activation phenotype has been studied during embryonic periods (Table 5). As  
22 for the findings in postnatal offspring, the results on embryonic microglia activation are divergent. For  
23 example, out of 6 studies that measured microglial density in embryonic MIA offspring, 3 studies  
24 measured no change while the other 3 studies detected increased microglial densities. Whilst other  
25 studies did not measure alterations in microglia density or morphology upon MIA, they measured

1 altered gene and protein expression of inflammatory mediators in embryonic brain or microglia  
2 (Cunningham *et al.* 2013, Pratt *et al.* 2013, Pont-Lezica *et al.* 2014, O'Loughlin *et al.* 2017, Schaafsma  
3 *et al.* 2017). Similar to studies measuring cytokine levels in the brain of postnatal MIA-offspring, also  
4 several studies analyzing embryonic brains measured cytokines in brain homogenates rather than in  
5 isolated microglia. In this review, to exclude the possible contribution of maternally derived cytokines,  
6 we consider only studies measuring cytokines at least 48 hours after the immune challenge. As such,  
7 time-dependent changes in the levels of IL10, TNF $\alpha$  and IL1 $\beta$  were detected (O'Loughlin *et al.* 2017).  
8 To measure changes in microglia specifically, Pont-Lezica *et al.* 2014 used genome-wide gene  
9 expression profiling of isolated embryonic microglia and found a differential gene expression of 3906  
10 genes upon maternal inflammation (Pont-Lezica *et al.* 2014). They found that several immune genes  
11 such as IL1 $\beta$ , C-C motif Chemokine ligand 4 (CCL4) and Nuclear factor (NF)-kB were upregulated while  
12 microglial genes involved in neuritogenesis such as Semaphorin (Sema)3c and Versican (Vcan) were  
13 downregulated (Pont-Lezica *et al.* 2014). Cunningham *et al.* 2013 performed immunohistochemical  
14 analyses on embryonic brain slices from rats exposed to LPS against markers indicative for different  
15 inflammatory activation states including inducible nitric oxide synthase (iNOS) and IL1 $\beta$  (pro-  
16 inflammatory state) or arginase-1 and IL-1RA (anti-inflammatory state) respectively. While the amount  
17 of microglia that expressed arginase-1 was not changed, the proportion of microglia expressing iNOS,  
18 IL1 $\beta$  or IL-1Ra was increased upon LPS administration (Cunningham *et al.* 2013). Similarly, repeated  
19 prenatal administration of LPS in mice increased mRNA levels for IL1 $\beta$ , IL6 and TNF $\alpha$  in isolated  
20 microglia 3 hours after the last LPS injection (Schaafsma *et al.* 2017). Furthermore, Pratt *et al.* 2013  
21 assessed both mRNA and protein levels of inflammatory mediators in isolated microglia from MIA-  
22 exposed embryonic brains. They found increased levels of IL6 mRNA whereas mRNA levels for IL1 $\beta$ ,  
23 TNF $\alpha$  and IL10 were unchanged. On the protein level, they found increased expression of IL1 $\alpha$ , IL4 and  
24 IL9 but not IL1 $\beta$ , IL6, IL10 or TNF $\alpha$  (among others) (Pratt *et al.* 2013). The fact that mRNA levels for IL6  
25 were increased while protein levels for this cytokine remained unchanged could be explained by  
26 differences in transcriptional and translational dynamics, as mentioned before. In contrast to these

1 findings, no change in microglial protein levels of inflammatory markers including iNOS, IL1 $\beta$  or Mac2  
 2 was detected in the hippocampus and cortex of embryos at different developmental stages upon  
 3 Poly(I:C) exposure at GD 11.5 (Smolders *et al.* 2015). Also repeated immune stimulation (at GD 11.5  
 4 and GD 15.5) did not affect embryonic microglia at GD 17.5 (Smolders *et al.* 2015).

5 **Table 5: Microglia alterations in embryonic MIA offspring**

Microglia alterations in embryonic MIA offspring					
Toxin/ Ref.	Species/ Dose/ Gestation Day	Age of Analysis	Regions analyzed	No change	Increase
Poly(I:C)  (Pratt <i>et al.</i> 2013)	C57BL6 mice/ 20mg/kg (i.9.)/ GD 12.5	GD 16.5	Whole brain	mRNA	mRNA
				IL1 $\beta$ , IL10, TNF $\alpha$	IL6
				Protein	Protein
				IL1 $\beta$ , IL2, IL3, IL5, IL6, IL7, IL10, IL12p40, IL12p70, IL13, IL15, IL17, IFN $\gamma$ , TNF $\alpha$ , ILF, G- CSF, VEGF, ...	IL1 $\alpha$ , IL4, IL9, GM-CSF, M-CSF
				Density	

<b>Poly(I:C)</b>	C57BL6 mice/	GD 11.5,	Cx, HPC	Density	
(Smolders <i>et al.</i>	20mg/kg (i.p.)/	GD 12.5,		IL1 $\beta$ , iNOS,	
2015)	11.5 and/or 15.5	GD 17.5		Mac2	
<b>LPS</b>	C57BL6 mice/	PND 0	HPC	Density	
(Roumier <i>et al.</i>	120 $\mu$ g/kg (i.p.)/				
2008)	GD 15.5				
<b>LPS</b>	CD-1 mice/ 8 $\mu$ g/kg	GD 18	Forebrain	% Iba+ cells	
(Le Belle <i>et al.</i>	(i.p.)/ GD 9.5				
2014)					
<b>LPS</b>	C57BL6 mice/	GD 17.5	HPC, Fimbria, CC,	Morphology	3906
(Pont-Lezica <i>et al.</i>	0.12 $\mu$ g/kg (i.p.)/		CgCx		differentially
2014)	GD 15.5				expressed
					genes
<b>LPS</b>	C57BL6 mice/	GD 18.5	Cx	Density and	
(Tronnes <i>et al.</i>	140 $\mu$ g/kg (i.p.)/			morphology	
2016)	GD 17.5			(larger cell body	
				and fewer and	
				shorter	
				processes)	
<b>LPS</b>	C57BL6 mice/	GD 17.5	Whole brain	mRNA	
(Schaafsma <i>et al.</i>	50 $\mu$ g/kg (i.p.)/	GD		IL6, IL1 $\beta$ , TNF $\alpha$	
2017)	15.5 – 17.5				
<b>LPS</b>	C57BL6 mice/	GD 16	Whole brain	IL1 $\beta$ , IL6, IL10	TNF $\alpha$ , IL10
	50 $\mu$ g/kg (i.p.)	GD 18		and/or TNF $\alpha$	TNF $\alpha$

(O'Loughlin <i>et al.</i> 2017)	GD 12	PND 0			IL6
<b>LPS</b> (Cunningham <i>et al.</i> 2013)	Rat/ 100µg/kg (i.p.)/ GD 15.5 – 16.5	GD 19.5	Cx	Morphology	iNOS, IL1β, IL1Ra
<b>IL6</b> (Gumusoglu <i>et al.</i> 2017)	CD-1 mice/ 100ng (i.p.)/ GD 12.5 – 14.5 3x/day	GD 14.5	Cx	Overall density	More multivacuolated microglia

1

2 **HPC: Hippocampus, Cx: Cortex, CgCx: Cingulate Cortex, CC: Corpus Callosum**

3 During rodent neurodevelopment, the brain environment substantially changes every day and  
4 microglia are likely to adapt accordingly and even contribute to these changes (Cunningham *et al.*  
5 2013, Matcovitch-Natan *et al.* 2016, Hanamsagar and Bilbo 2017). As a consequence, potential MIA-  
6 induced alterations in microglia functions could be transient and its detection highly dependent on  
7 when and where microglia phenotypes are assessed. This could in part explain some of the  
8 controversial findings in microglia phenotypes described. For example Tronnes *et al.* 2016 found an  
9 increased microglia density and altered microglia morphology in the somatosensory cortex 24 hours  
10 after MIA at GD 17.5 (Tronnes *et al.* 2016), whilst no evidence of altered microglia density was detected  
11 in the fetal cortex and hippocampus 24 hours after maternal injection of Poly(I:C) at GD 11.5 (Smolders  
12 *et al.* 2015). Again direct comparison between these two studies is problematic due to the difference  
13 in MIA paradigm and time of MIA induction. Nevertheless, others detected no changes in fetal  
14 microglia density or morphology 48-72 hours post-MIA induction (Cunningham *et al.* 2013, Pratt *et al.*  
15 2013, Pont-Lezica *et al.* 2014, Smolders *et al.* 2015) whilst one study described an increase in the  
16 percentage of Iba1 positive cells 9 days post-LPS exposure (Le Belle *et al.* 2014). A similar controversy

1 arises when microglial protein expression is assessed. For example, while Cunningham *et al.* 2013  
2 found increased protein expression of iNOS and IL1 $\beta$ , 72 hours after maternal LPS administration at  
3 GD 15.5 and GD 16.5, Smolders *et al.* 2015 found no change in the expression of these proteins 5, 24,  
4 48 or 144 hours after maternal Poly(I:C) exposure at GD 11.5 or after a subsequent exposure at GD  
5 15.5 (Cunningham *et al.* 2013, Smolders *et al.* 2015).

6 Taken together, clear conclusions cannot be drawn from the current data available on embryonic  
7 microglia phenotypes after MIA, firstly due to the omnipresent variety in the protocols applied (a  
8 similar phenomenon is seen in the studies assessing postnatal microglia phenotypes) and secondly due  
9 to the dynamic changes of microglia phenotypes during normal early development, which necessitates  
10 highly similar protocols of MIA induction and embryonic time points of analyses. However, as  
11 suggested by the study of Mattei *et al.* 2017, it can be concluded that embryonic microglia are able to  
12 sense and respond to MIA, causing long-term changes in their transcriptomic signature. This might  
13 indicate that the early embryonic insult deviates the phenotypical track of microglia, which in turn  
14 could also affect the development of other brain cells. Therefore, the characterization of MIA-induced  
15 changes in embryonic microglia at different developmental stages is crucial in order to understand  
16 how microglia could alter normal developmental trajectories that precipitate into the neuronal and  
17 behavioral deficits detected in MIA.

18

#### 19 **4. Age, sex and brain region might affect microglial phenotype upon MIA**

20 In the previous section, we discussed the influence of the specific read-out to assess microglia  
21 activation (i.e. density, morphology, cytokine production and marker expression) on the precise  
22 outcome of the study. However, even when considering the same read-out, factors that are indirectly  
23 related to the analytical method will contribute to variability between studies. These factors include  
24 physiological constraints that alter the microglial phenotype such as age, sex and brain region.

#### 1 **4.1 Brain region-specific changes in microglia of MIA offspring**

2 In the normal brain, several microglial properties appear to be highly dependent on the specific brain  
3 region, including density, morphology, membrane properties and transcriptional profiles, possibly  
4 reflecting a different set of tasks in different brain regions (Schwarz *et al.* 2012, Yang *et al.* 2013,  
5 Grabert *et al.* 2016, De Biase *et al.* 2017). Moreover, a physiological function of microglia that received  
6 much attention in recent years, i.e. synaptic refinement, also appears to be region-dependent. In the  
7 hippocampus, elimination of synapses is dependent on CX3CR1 as mice lacking this receptor displayed  
8 increased hippocampal dendritic spine density accompanied by synaptic characteristics reminiscent of  
9 an immature connectivity and weak synaptic transmission (Paolicelli *et al.* 2011, Zhan *et al.* 2014). On  
10 the other hand, synaptic remodeling in the visual system was shown to be dependent on the  
11 complement system (in the lateral geniculate nucleus) and on P2Y12 (in the visual cortex) (Schafer *et*  
12 *al.* 2012, Sipe *et al.* 2016, Schechter *et al.* 2017). Hence, it should be of no surprise that environmental  
13 insults could affect microglia differently in different brain regions. In the context of MIA, several studies  
14 found changes in microglial density in one region while other regions were comparable to control mice  
15 upon Poly(I:C)-induced MIA (Juckel *et al.* 2011, Manitz *et al.* 2013, Mattei *et al.* 2014, Van den Eynde  
16 *et al.* 2014, Hadar *et al.* 2016, Hui *et al.* 2018).

17

#### 18 **4.2 Age of MIA offspring**

19 Although schizophrenia and ASD share a subset of risk factors and symptoms (such as social interaction  
20 and communication deficits), they differ greatly in the age of disease onset. While ASD is usually  
21 diagnosed during childhood, schizophrenia typically emerges around adolescence. Similarly, the  
22 emergence of behavioral deficits appears to be age-dependent in the MIA model, which is effectively  
23 used to study the etiology of both ASD and schizophrenia. While deficits in prepulse inhibition, latent  
24 inhibition and amphetamine-induced activity are usually observed in adult MIA offspring (e.g.  
25 (Piontkewitz *et al.* 2011, Hadar *et al.* 2015, Fujita *et al.* 2016, Han *et al.* 2016)) (working) memory and



1 social interaction deficits can readily be detected in pubescence (e.g. (Malkova *et al.* 2012, Fujita *et al.*  
2 2016, Han *et al.* 2016)). Moreover, on the neuroanatomical level, decreases in hippocampal volume  
3 could be observed in pubescent MIA offspring while accelerated cortical thinning was only detectable  
4 in adulthood (Piontkewitz *et al.* 2011, Crum *et al.* 2017).

5 In the normal brain, microglial properties such as density and morphology change substantially over  
6 time (e.g. (Schwarz *et al.* 2012, Swinnen *et al.* 2013)). Also the transcriptional profile of microglia is  
7 highly dependent on age, possibly allowing microglia to perform specific functions at specific stages  
8 (Grabert *et al.* 2016, Matcovitch-Natan *et al.* 2016, Thion *et al.* 2018). Finally, it was recently shown  
9 that the influence of the microbiome (as will be discussed later on in this review) on the microglial  
10 transcriptome is different according to age (with smaller influence in early development) (Thion *et al.*  
11 2018)).

12 Consequently, effects of MIA on microglia might also be dependent on the age at which analysis was  
13 performed. Increased microglial densities were found in the hippocampus of Poly(I:C)-induced MIA  
14 offspring between PND 2 and 30, which were no longer present in adulthood (Manitz *et al.* 2013, Li *et*  
15 *al.* 2014) while increased striatal microglia density was only observed in PND 100 offspring (Manitz *et*  
16 *al.* 2013). Furthermore, Esslinger *et al.* 2016 described changes in the expression of several microglial  
17 surface markers in whole-brain microglia from 30-day-old Poly(I:C)-MIA offspring, a phenotype that  
18 was lost when the offspring reached PND 100. A possible explanation for the loss of this phenotype  
19 could be the fact that microglia function changes during development – from progenitor pool  
20 maintenance in early brain development to synaptic remodeling during adolescence and adult brain  
21 homeostasis – and that the changes observed in microglia are related to the age-specific function of  
22 microglia at that specific point in time (Mosser *et al.* 2017).

### 23 **4.3 Sex of MIA offspring**

24 Neurodevelopmental disorders such as schizophrenia and ASD have a strong gender-bias, with a higher  
25 prevalence in males than in females (Abel *et al.* 2010, Mukherjee 2017). Although only a handful of

1 studies directly compared the effects of MIA on behavior in both male and female offspring, most find  
2 a more severe behavioral deficit in males compared to females (Taylor *et al.* 2012, Naviaux *et al.* 2013,  
3 Xuan and Hampson 2014, Meehan *et al.* 2017, Hui *et al.* 2018). With regard to MIA studies investigating  
4 the effect on microglia, about one third of studies did not report on whether they analyzed males or  
5 females (e.g. (Roumier *et al.* 2008, Hsiao *et al.* 2012, Cunningham *et al.* 2013, Pratt *et al.* 2013)) and  
6 two thirds of studies used either males or a mix of males and females (not analyzed separately) (e.g.  
7 (Van den Eynde *et al.* 2014, Giovanoli *et al.* 2015, Hadar *et al.* 2016, Paylor *et al.* 2016)). Yet, several  
8 recent reports indicate substantial differences between microglial properties of both sexes, including  
9 microglia density and morphology, phagocytic activity and migration (Schwarz *et al.* 2012, Nelson *et*  
10 *al.* 2017, Weinhard *et al.* 2017, Yanguas-Casas *et al.* 2018). Also on the transcriptional level, differences  
11 have been described between male and female microglia. While the microglial transcriptome is similar  
12 during development, sexual dimorphism increases in adulthood (with female microglia in a more  
13 immune-activated state) (Hanamsagar *et al.* 2017, Thion *et al.* 2018). Moreover, the effects of  
14 postnatal immune challenge or of pre- and postnatal microbiome composition on the microglial  
15 transcriptome was dependent on sex (Hanamsagar *et al.* 2017, Thion *et al.* 2018). Hence, MIA might  
16 differentially affect male versus female microglia and thus result in divergent behavioral outcomes.

17 To our knowledge, only 3 studies directly compared the effects of MIA on microglia between male and  
18 female offspring. As such, Manitz *et al.* 2016 and Esslinger *et al.* 2016 showed sex-specific alterations  
19 in the expression of several proteins associated with microglial activation, including CD45, CD54,  
20 CD124 and CD206 ((Esslinger *et al.* 2016, Manitz *et al.* 2016)) in whole-brain lysates of Poly(I:C)-MIA  
21 offspring. Hui *et al.* 2018 recently also showed more pronounced effects of MIA (induced by Poly(I:C)  
22 on microglial distribution and arborization in the hippocampus of male offspring compared to females  
23 (Hui *et al.* 2018). Of interest, the studies described in this section all used Poly(I:C) to induce maternal  
24 inflammation when comparing the effects of MIA on microglia in different brain regions, between  
25 sexes or at different ages. Besides O'Loughlin *et al.* 2017, who identified microglial morphological

1 changes at 3 different ages, no other studies that used LPS investigated the influence of the  
2 aforementioned factors.

3 Failure to report on the sex of the offspring examined is mostly prevalent in studies analyzing  
4 embryonic microglia, as sex determination by eye is not possible at this age. However, as done by  
5 Hsueh *et al.* 2017, genotyping of embryonic samples for the presence of the sex-specific SRY gene can  
6 readily distinguish between males and females at embryonic stages (Hsueh *et al.* 2017). Hence, the  
7 fact that microglia are measured at embryonic ages should not be a limitation to investigate gender  
8 differences.

9

## 10 **5. MIA-model associated factors can influence the study outcome**

11 As discussed in the previous section, different studies used different methodologies to determine  
12 microglia activation upon MIA, which could have led to the contradicting results on the effect of MIA  
13 on microglia. However, another notable difference between studies is the exact method used to induce  
14 MIA. Both Poly(I:C) and LPS are administered at several doses and at different gestational ages, all of  
15 which are factors that could contribute to the variation in results that have been found by different  
16 studies. Moreover, lab specific factors such as housing conditions and food composition are likely to  
17 play an important role in the diversity between studies. In the following sections, we will discuss the  
18 possible influence of these factors on the outcome of studies determining microglia activation.

### 19 **5.1 Poly(I:C) vs LPS**

20 The two most commonly used agents to induce MIA in rodents are LPS and Poly(I:C). MIA studies  
21 determining microglial alterations at postnatal or adult ages mostly used Poly(I:C) and only a few  
22 induced MIA with LPS. Regardless of which immune stimulant was used however, several studies  
23 suggest microglia “activation” in the adult MIA offspring while just as many studies find no evidence  
24 for changes in microglia. In contrast, studies assessing microglia “activation” at embryonic stages

1 mostly used LPS to induce MIA with the exception of Pratt *et al.* 2013 and Smolders *et al.* 2015, who  
2 used Poly(I:C). Both Poly(I:C) and LPS elicit their effects via the toll like receptor (TLR) family, where  
3 Poly(I:C) signals via the TLR3 and LPS via TLR4. Unlike TLR4, which is located at the cellular membrane,  
4 TLR3 is localized to endosomal membranes. Although activation of both TLR3 and TLR4 seems to result  
5 in similar, but not equal, intracellular and cytokine responses, the precise set of immune mediators  
6 released by both immune activating agents has, to our knowledge, not been described. However, it  
7 seems likely that the different immune stimulants elicit the production and secretion of different  
8 subsets of cytokines (Reisinger *et al.* 2015, Bilbo *et al.* 2017). Indeed, specific cytokines have been  
9 attributed to play a critical role in both the Poly(I:C)-based and LPS-based MIA model. In 2007, Smith  
10 *et al.* showed that systemic injection of recombinant IL6 was able to evoke similar behavioral deficits  
11 as Poly(I:C) and that simultaneous injection of IL6 blocking antibody could prevent the MIA-induced  
12 behavioral changes (Smith *et al.* 2007). Complementary to this, Wu *et al.* 2017 showed that deletion  
13 of the IL6 receptor specifically in the placenta could prevent MIA-induced deficits in the offspring (Wu  
14 *et al.* 2017). Furthermore, it was shown that IL17a acts downstream of IL6 as a systemic injection of  
15 IL17a-antibody before Poly(I:C)-induced MIA was able to prevent altered ultrasonic vocalizations in  
16 MIA offspring (Choi *et al.* 2016). In addition to schizophrenia and ASD related symptoms, Pineda *et al.*  
17 2013 also showed the importance of specific cytokines for epilepsy, a well-known co-morbidity in ASD  
18 patients. While the presence of only IL6 in maternal blood was sufficient to induce social impairment  
19 in the offspring, a combination of IL6 and IL1 $\beta$  was required to increase susceptibility to seizures  
20 (Pineda *et al.* 2013). In contrast to IL6, blocking IL1 $\beta$  upon Poly(I:C) administration did not improve  
21 social deficits (Pineda *et al.* 2013). Although the previous studies described the importance of IL6 and  
22 IL17a in mediating the effects of Poly(I:C)-induced MIA on the offspring, IL1 was shown to be crucial  
23 for LPS-mediated effects (Girard *et al.* 2010) as co-administration of an IL1 receptor antagonist could  
24 alleviate LPS-induced placental damage, maintain microglia density and protect against motor  
25 dysfunction in the offspring.

1 Beside the classical activation of TLR4, LPS was recently also shown to induce activation of transient  
2 receptor potential (TRP) channels in a TLR4-independent manner in airway epithelial cells and  
3 nociceptive sensory neurons (Meseguer *et al.* 2014, Alpizar *et al.* 2017). Activation of TRP channels in  
4 airway epithelial cells was speculated to be caused by mechanical perturbations induced by LPS in the  
5 plasma membrane and induced the production of bactericidal nitric oxide (Alpizar *et al.* 2017).  
6 Whether LPS also activates TRP channels in microglia is not known but, intriguingly, increased numbers  
7 of microglia expressing iNOS and IL1 $\beta$  were detected when acute embryonic brain slices were  
8 challenged with LPS whereas exposure to Poly(I:C) and recombinant IL6 failed to do the same  
9 (Smolders *et al.* 2015). These results raise the question whether LPS might have more potent effects  
10 both on microglia and other cells due to its additional activation of TRP channels.

11 In addition to specific cytokines induced by either Poly(I:C) or LPS, a different intensity of the immune  
12 response (either by administration of a higher dose or by repeated exposure) provoked by both  
13 immune stimulants could explain the differential effects of these 2 agents. Indeed, dose-response  
14 studies by Shi *et al.* 2003 and Meyer *et al.* 2005 showed that increasing doses of Poly(I:C) correlated  
15 with increased levels of cytokines in maternal blood and with increased severity of behavioral deficits  
16 in the offspring while increasing doses of LPS were able to induce abortion (Hsueh *et al.* 2017). As  
17 different batches of LPS and Poly(I:C) can differ in the intensity of immune response they induce, dose-  
18 response studies are crucial and need to be performed. Moreover, the synthetic Poly(I:C) comes in  
19 different forms, low and high molecular weight or with added sodium salt, depending on the company,  
20 which could influence the precise dose. Hence, a clear statement on which Poly(I:C) was used and the  
21 exact dose should be included in every publication. Finally, a comparative study between LPS and  
22 Poly(I:C) addressing both classical immune activation and developmental functions of embryonic  
23 microglia would be of great value.

## 24 **5.2 Timing of MIA induction**

1 Besides the immune activating agent used to induce MIA, the embryonic age at which MIA is induced  
2 varies greatly between studies, ranging from early to mid (GD 9, GD 11, GD 12) and late gestation (GD  
3 17). Intriguingly, studies comparing the behavioral deficits in offspring exposed to MIA during different  
4 gestational periods have shown that certain, but not all, behavioral phenotypes show a gestational  
5 dependency ((Meyer *et al.* 2006) and others). The precise mechanisms underlying this dependency  
6 remain to be fully identified (as discussed in detail in (Meyer *et al.* 2007)). The stage and ongoing  
7 processes of fetal development, as well as placental development have been suggested to play an  
8 important role. Besides this, microglial developmental stages could be perturbed differently  
9 depending on when MIA is induced, which could result in different perturbations of  
10 neurodevelopmental processes. Indeed recent findings of whole genome expression analyses of  
11 microglia isolated at different embryonic ages could identify distinct developmental stages of microglia  
12 (GD 10.5 - 14.5, GD 14.5 – PND 9 and PND 28) (Matcovitch-Natan *et al.* 2016). This could explain the  
13 findings described by Lai *et al.* 2013 where they detected an age-specific and activator-dependent  
14 activation profile of isolated, cultured microglia (Lai *et al.* 2013, Matcovitch-Natan *et al.* 2016). In line  
15 with this, Hanamsagar *et al.* 2017 identified a specific developmental maturation and immune  
16 reactivity profile of microglia in both mice and humans, which was shown to be gender- and age-  
17 dependent (Hanamsagar *et al.* 2017). Hence, it seems plausible that microglia could be differentially  
18 perturbed depending on when MIA was induced which leads to differential changes in brain  
19 developmental processes and thus subtle changes in behavioral deficits later in life.

20 In addition to the immediate effects of MIA on microglia functions and early neurodevelopment,  
21 prenatal immune stimulation might prime embryonic microglia resulting in an exacerbation of their  
22 response to stimulants later in life (for example stress during childhood or puberty) and as such cause  
23 a deregulation in critical processes of brain maturation (Eggen *et al.* 2013, Knuesel *et al.* 2014,  
24 Slusarczyk *et al.* 2015, Giovanoli *et al.* 2016). Such priming effects were shown by Krstic *et al.* 2012  
25 where an increased CD68 reactivity and changes in microglial morphology was observed in aged  
26 offspring that were exposed to two Poly(I:C) immune challenges, once during prenatal development

1 (GD 17) and once during adulthood (Krstic *et al.* 2012). Giovanoli *et al.* 2013 and 2016 described  
2 increased expression of CD68 and CD11b together with an enlargement of the microglial cell soma in  
3 adolescent MIA offspring that was exposed to unpredictable stress during postnatal development  
4 (Giovanoli *et al.* 2013, Giovanoli *et al.* 2016). Intriguingly, they further showed that minocycline  
5 treatment – a broad-spectrum tetracycline antibiotic that was shown to inhibit microglia activation –  
6 during postnatal stress prevented the offspring to develop behavioral deficits (Giovanoli *et al.* 2016),  
7 pointing towards the importance of proper microglia function during postnatal periods for brain  
8 maturation, neuronal function and behavior.

### 9 **5.3 Housing, food, microbiome, strain and species effect on MIA outcome**

10 As briefly touched upon in the introduction, several lab-specific factors that are not directly related to  
11 the protocol used to induce MIA in rodents might influence the response to MIA and contribute to the  
12 variation in study outcome we observe.

13 The two rodent species that are by far mostly used in the lab are mice and rats. However, it was  
14 recently shown by Lam *et al.* 2017 that the microglial response to inflammatory stimuli is different  
15 between these species (Lam *et al.* 2017). Even within a rodent species, different **strains** are being used  
16 to study the effect of MIA on microglia. A handful of studies reported a different vulnerability of inbred  
17 mouse strains, with different genetic background, to the effects of MIA (Schwartzter *et al.* 2013, Babri  
18 *et al.* 2014, Morais *et al.* 2018). Alterations in ultrasonic vocalizations, social interaction and repetitive  
19 behavior were more pronounced in BTBR mice compared to C57BL6 mice (Schwartzter *et al.* 2013).  
20 Similarly, maternal inflammation in NMRI or NIH Swiss mice induced more pronounced anxiety and  
21 depression-like behavior compared to C57BL6 mice (Babri *et al.* 2014, Morais *et al.* 2018).

22 Interestingly, Morais *et al.* 2017 showed that the increased vulnerability of NIH Swiss mice to effects  
23 of MIA on the offspring was accompanied by an increased gut permeability in adulthood, a defect that  
24 has previously been associated with **microbiome** dysregulation (Hsiao *et al.* 2013, Morais *et al.* 2018).  
25 Accumulating evidence suggests an important effect of microbiome composition, both from the

1 mother and the offspring, on the development and homeostasis of microglia and the behavioral  
2 outcome of MIA. Adult germ-free mice or mice with limited microbiota complexity displayed global  
3 defects in microglia leading to an impaired innate immune response (Frick *et al.* 2013, Matcovitch-  
4 Natan *et al.* 2016). Also in prenatal stages, absence of maternal gut microbiota resulted in dysregulated  
5 microglia transcriptomic profiles, altered microglial colonization of the embryonic brain (i.e. increased  
6 microglial density and excessive ramification) and increased chromatin accessibility (Thion *et al.* 2018).

7 The importance of microbiome composition in the context of MIA has also recently been addressed.  
8 Eradication of the vancomycin-susceptible segmented filamentous bacteria (SFB) prior to MIA  
9 induction prevented the emergence of behavioral deficits in the offspring (Kim *et al.* 2017).  
10 Additionally, maternal infection in C57BL6 mice obtained from Taconic Biosciences (which contain  
11 intestinal SFB) induced behavioral abnormalities in the offspring while the same protocol applied to  
12 C57BL6 mice from Jackson Laboratories (which are devoid of intestinal SFB) failed to elicit a behavioral  
13 phenotype (Kim *et al.* 2017). Finally, MIA was shown to induce a dysregulation of the offspring's  
14 microbiome which in turn was associated with behavioral symptom severity (Hsiao *et al.* 2013).  
15 Treatment of MIA offspring with microbiota partially prevented these gut alterations along with  
16 stereotypical and anxiety-like behavior and deficits in sensorimotor gating (Hsiao *et al.* 2013).

17 Another variable between labs is the **composition of the food** provided to rodents. Nevertheless, the  
18 composition of different food pellets can differ dramatically and influence the outcome of MIA studies.  
19 One important aspect of rodent food pellets is the amount of anti-inflammatory dietary omega 3  
20 polyunsaturated fatty acids (PUFA) relative to the amount of pro-inflammatory omega 6 PUFAs. The  
21 importance of dietary omega 3 PUFA for brain development has been documented in rodents:  
22 deficiency during development in rodents leads to deficits in cognition, vision and a wide array of  
23 behaviors (Fedorova *et al.* 2009, Brenna 2011, Weiser *et al.* 2015) while feeding mice with diets high  
24 in omega 6 PUFA during gestation produces autistic-like features in offspring (Jones *et al.* 2013). In the  
25 context of MIA, Li *et al.* 2015 and Weiser *et al.* 2016 showed that omega 3 PUFA-enriched or



1 docosahexaenoic acid-supplemented diet could prevent the onset of behavioral deficits in MIA  
2 offspring (Li *et al.* 2015, Weiser *et al.* 2016). Given the anti-inflammatory properties of omega 3 PUFAs,  
3 it seems plausible that the preventive effects could partially be explained by reduced microglial  
4 activation.

5 Finally, **housing conditions** can influence the effect of MIA on offspring's microglial and behavioral  
6 phenotype. In the study of Buschert *et al.* 2016, MIA offspring was either housed in standard conditions  
7 or in an environmentally enriched cage. Although no typical MIA-induced behavioral changes were  
8 observed, prenatal MIA reduced overall exploration (in the sociability test) in the environmentally  
9 enriched group, while it failed to do so in the standard housed animals as ~~exploration~~. Similarly, a  
10 decrease in cortical microglial density upon MIA (which is in sharp contrast to the studies discussed in  
11 this review describing either unchanged or increased density) could only be observed in mice housed  
12 in an enriched cage, while both NaCl and Poly(I:C)-exposed mice housed in a standard cage displayed  
13 decreased microglial density compared to the environmental enrichment group. The increase in  
14 microglial density, which is often interpreted as a sign of inflammation, upon housing in an enriched  
15 environment seems to contradict the findings of an anti-inflammatory effect of an enriched  
16 environment in disease contexts (including Alzheimer and Depression) (Williamson *et al.* 2012, Chabry  
17 *et al.* 2015, Xu *et al.* 2016). However, as shown by Ziv *et al.* 2006 and speculated by Buschert *et al.*  
18 2016, these microglia might be in a neuroprotective rather than an inflammatory state (Ziv *et al.* 2006,  
19 Buschert *et al.* 2016). Taken together, these findings suggest that standard housing conditions can  
20 mask behavioral deficits and possibly microglial alterations induced by MIA.

21 In conclusion, we want to stress that authors should mention as much methodological details as  
22 possible in the materials and methods section concerning strain, housing, food etc., as they all have  
23 been shown to be relevant for the exact outcome of MIA studies and as a consequence possibly for  
24 the microglial response to MIA.

25

## 1 **6. Alterations in microglia beyond dysregulated immune functions**

2 Over the last decade there has been a substantial shift in our understanding of the functional role of  
3 microglia with regard to their non-immunological functions. Microglia are the first glial cells present in  
4 the developing brain and have been attributed to play a role in neurogenesis, angiogenesis,  
5 myelination, synaptogenesis and synaptic pruning (Bilimoria and Stevens 2015, Reemst *et al.* 2016).  
6 Although these functions are considered “non-immune” functions, some of them still rely on immune  
7 components, e.g. synaptic pruning has been shown to depend on the complement system (Hong *et al.*  
8 2016). The fact that microglia originate from primitive macrophages and exert classical immune  
9 functions in response to insults of the CNS in the adult brain has led to the assumption that embryonic  
10 microglia exert similar roles in the developing brain. However, although equipped with immune related  
11 machinery, their functional contribution during development was found to exceed that of classical  
12 immune functions.

13 In support of this, microglia depletion during the first two weeks after birth was shown to result in  
14 long-lasting behavioral deficits (VanRyzin *et al.* 2016). Furthermore, transcriptomic studies showed  
15 that microglia are able to adapt according to regional cues, which translates in a regional and age-  
16 dependent density, morphology, surface molecule expression and transcriptome profile of microglia  
17 (Hart *et al.* 2012, Schwarz *et al.* 2012, Butovsky *et al.* 2014, Grabert *et al.* 2016, De Biase *et al.* 2017,  
18 Mosser *et al.* 2017). In fact, microglia might adjust their functional phenotype as a response to  
19 developmentally changing factors in the neuro-environment to be able to perform their specific  
20 developmental task (Mosser *et al.* 2017). Hence, although studies investigating classical activation of  
21 microglia upon MIA are highly valuable, it might be more appropriate to look at gene profiles involved  
22 in the neurodevelopmental function of microglia, combined with assessing the related microglial  
23 functions during brain development. Indeed, recent studies point to MIA-induced changes in the  
24 microglial epigenome (Kaminska *et al.* 2016, Nardone and Elliott 2016) and transcriptome concerning  
25 genes involved in the developmental functions of microglia, such as genes involved in synaptogenesis

1 and neuronal network formation (Pont-Lezica *et al.* 2014, Squarzoni *et al.* 2014, Matcovitch-Natan *et*  
2 *al.* 2016, Mattei *et al.* 2017). Matcovitch-Natan *et al.* described that microglia from mice subjected to  
3 MIA and analyzed from GD 14 to early postnatal weeks were transcriptionally shifted toward a more  
4 advanced developmental stage (Maticovitch-Natan *et al.* 2016). This implies that MIA renders microglia  
5 more “mature”, which is in accordance with a recent microglial transcriptomics study suggesting an  
6 accelerated development of these cells in autism patients (Hanamsagar *et al.* 2017).

7 In support of altered neurodevelopmental microglial functions upon MIA, several reports describe  
8 increased phagocytosis of neuronal progenitor cells (Cunningham *et al.* 2013), a decrease in the  
9 outgrowth of dopaminergic axons (Squarzoni *et al.* 2014) and defasciculation of dorsal callosal axons  
10 (Pont-Lezica *et al.* 2014) in the embryonic brain of MIA offspring. In the latter case, mice lacking DNAX-  
11 Activation Protein 12 (DAP12), a transmembrane protein that together with Triggering receptor  
12 expressed on myeloid cells 2 (TREM2) forms an immunoreceptor signaling complex and is associated  
13 with Nasu-Hakola disease, displayed similar deficits as observed after MIA. Moreover, MIA led to  
14 transcriptional alterations in microglia with regard to genes involved in growth of neurites,  
15 underscoring a disturbed developmental function of microglia after MIA (Pont-Lezica *et al.* 2014). Until  
16 now, conclusive experimental evidence for a direct, causative role of microglia in neurodevelopmental  
17 disorders after MIA is lacking. However, a recent study by Mattei *et al.* 2017 showed that MIA-induced  
18 changes in the microglial transcriptome and phagocytic function were accompanied by behavioral  
19 abnormalities (Mattei *et al.* 2017). Most interestingly, treatment of adult MIA offspring with  
20 minocycline rescued the transcriptional, functional and behavioral deficits. This study is among the  
21 first to point in the direction of dysfunctioning microglia as the culprits in inducing  
22 neurodevelopmental disorders upon MIA. Furthermore, disturbance of microglia-related proteins in  
23 humans such as Colony stimulating factor 1 receptor (CSF1r), TREM2, Methyl CpG binding protein 2  
24 (MecP2), CD33, Interferon regulatory factor 8 (Irf8), P2x7r, DAP12 and Complement component 4 (C4)  
25 are associated with an increased likelihood to develop a plethora of neurological diseases, including  
26 schizophrenia and possibly autism ((Sekar *et al.* 2016) and reviewed in (Frost and Schafer 2016)). These

1 data suggest that disturbance of specific functions of microglia during neurodevelopment correlate  
2 with disease outcome.

3 Taken together, microglia display specific and constantly changing transcriptional signatures  
4 throughout development and these profiles appear to be altered upon MIA. Although conclusive  
5 evidence to pinpoint microglial alterations as the cause for neurodevelopmental disorders is missing,  
6 accumulating evidence strongly suggests at least a partially shared causative role for microglia in MIA-  
7 induced cognitive and behavioral deficits.

8

## 9 **7. Conclusion**

10 In the search for evidence of altered microglia function as mediators for the neurological deficits  
11 caused by MIA, a lack of consistency is encountered. Several factors might explain these contradictory  
12 results and thereby add complexity to the comparison of different MIA studies with regard to microglial  
13 alterations. In this review, we addressed this issue of variability in the field and here we suggest  
14 guidelines that might result in convergence on findings with regard to the effects of MIA on microglia.  
15 On one hand, the exact protocol used to induce MIA in rodents differs substantially between studies,  
16 using different immune stimulants (mostly LPS or Poly(I:C)) at different doses and administration  
17 routes and administered at various gestational time points. These factors will result in a different  
18 environment to which the embryo is exposed and might differentially affect microglia. First, we want  
19 to urge researchers to fully state all experimental details (such as animal strain, housing conditions,  
20 food, sex, etc.) when reporting on the outcome of MIA studies. Secondly, we suggest to perform dose-  
21 response studies of the immune stimulant used before the start of the study, in order to better control  
22 the inflammatory environment that is created. Although behavioral testing would be the most ideal  
23 read-out for these dose-response studies, measurements of cytokines such as IL6 or IL17a in maternal  
24 serum might provide the minimal amount of information on the extent of maternal inflammation.

1 Finally, care should be taken to use the least invasive and stressful administration route, as stress could  
2 be an additional confounding factor for the outcome of the experiments.

3 On the other hand, substantial variability could be caused by the precise readout to assess “microglia  
4 activation” with respect to their immunological function, such as microglia density, morphology or  
5 immune marker expression, but also by the time point and brain region where microglia were  
6 analyzed. While many studies used only one of these read-outs to analyze the effect of MIA on  
7 microglia, we suggest to use a combination of several complementary methods to better define the  
8 precise changes observed in microglia. Moreover, although these relatively crude measures can aid  
9 our understanding of the role of microglia in MIA models to some extent, they might not be suitable  
10 to detect less obvious changes in microglia function related to developmental processes. Therefore,  
11 we believe future research on immune disturbance of neurodevelopment will benefit from a holistic  
12 approach in which developmental functions of microglia are studied using a combinatorial approach –  
13 i.e. using readouts at transcriptional, protein and functional level – to study both intrinsic microglial  
14 characteristics and the neuron-microglia interaction.

15

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19

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