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Investigating the role of microglia in neural development and synaptic maintenance

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INVESTIGATING THE ROLE OF MICROGLIA IN NEURAL DEVELOPMENT AND SYNAPTIC MAINTENANCE

by

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DEDICATION

I would like to dedicate this work to my mom, who inspired me to pursue science and was always there for me.

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INVESTIGATING THE ROLE OF MICROGLIA IN NEURAL DEVELOPMENT AND SYNAPTIC MAINTENANCE

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ABSTRACT

Maternal immune activation (MIA) disrupts the central innate immune system during a critical neurodevelopmental period. Microglia are the primary innate immune cells in the brain and can mediate neurodevelopment, but the direct influence of microglia on the MIA phenotype remains largely unknown. Here, we show that MIA can lead to long-lasting effects on microglial phenotype, neuronal circuitry, and behaviors. Transcriptomic analysis revealed aberrant expression of neurogenic genes in MIA microglia. We found that microglia repopulation by colony-stimulating factor receptor 1 (CSF1R) inhibition reversed MIA-induced social deficits and corrected expression of the newly identified MIA-associated neuritogenic molecules in microglia. *In vitro* whole-cell patch-clamp recording and immunohistochemistry revealed that microglia repopulation restored MIA-induced changes in intrinsic excitability, dendritic spine density, and microglia-neuron interactions of layer V intrinsically bursting pyramidal neurons in the prefrontal cortex. Maternal inflammation therefore alters microglial phenotypes and changes neuronal functions by mediating microglia-neuron interactions. We found that Wingless-related MMTV integration site 5a (WNT5a) is a critical regulator of this microglia-neuron communication. Studies have shown that the neurotrophic factor

WNT5a plays a critical role in neurodevelopment, and here we demonstrate that WNT5a is one of the neuritogenic genes significantly upregulated in embryonic MIA microglia. We showed using microarray analysis that the microglial secretome can promote neural stem cell differentiation through various pathways, including Wnt pathways. Live imaging of neuron-microglia co-culture demonstrated that microglia enhanced neurite development and dendritic spine density and that this was diminished by microglial *Wnt5a* silencing using siRNA transfection. Multi-electrode array recordings revealed that microglia co-culture increased spontaneous neuronal firing rate. Thus, microglia can secrete WNT5a and regulate dendritic spine development, maintenance, and neural circuitry. These results indicate that altered expression of microglial WNT5a due to pathogenic states such as inflammation can lead to abnormal neuronal activity. To further elucidate microglia biology, we developed an inducible immortalized murine microglial cell line using a tetracycline expression system. The addition of doxycycline can induce rapid cell proliferation for the expansion of cell colonies. Upon withdrawal of doxycycline, this monoclonal microglial cell line can differentiate and resemble *in vivo* microglia physiology as assessed by expression of microglial genes, innate immune response, chemotaxis, and phagocytic capabilities. This cell line becomes a convenient and useful method to study microglia *in vitro*.

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LIST OF ABBREVIATIONS

AP……...…….action potential

ASD…….….autism spectrum disorder

ATP…….…….……adenosine triphosphate

BAM….…….…….……border associated macrophages

BRDU ..... bromodeoxyuridine

CLEC7A……...…….C-type lectin domain family 7

CNS……...central nervous system

CR3…….....complement component 3 receptor

CSF-1R…….colony stimulating factor 1 receptor

CX3CR1……c-x3-c motif chemokine receptor 1

EDU.....5-ethynyl-2′-deoxyuridine

EMP…….……..erythromyeloid progenitors

FACS……...fluorescence-activated cell sorting

FCRLS……..……. fc receptor-like s, and scavenger receptor

GABA……..gamma aminobutyric acid

GWAS……...….genome-wide association studies

IB..... intrinsically bursting

IBA1……...……...…..…ionized calcium binding adaptor molecule 1

IFN-γ.....interferon gamma

IL.....interleukin

IPSC.....inhibitory postsynaptic current

ISI……..……..……..……..…… inter-spike interval

LPS…….lipopolysaccharides

MeCP2……..…… methyl-CpG binding protein 2

MG-REP……..microglia repopulation

MHC…….……..……..……major histocompatibility complex

MIA…….……maternal immune activation

mPFC…….……..medial prefrontal cortex

mRNA.....messenger RNA

NRP1……..…… neuropilin 1

P2RY12……..……..purinergic receptor P2Y G-protein coupled 12

PLX.....Plexxikon (CSF1R inhibitor)

poly(I:C)……..polyinosinic:polycytidylic acid

PSD……postsynaptic density

PUFA.…… …….……..……polyunsaturated fatty acids

qRT-PCR……..……..……..quantitative real time polymerase chain reaction

RS..... regular Spiking

SALL1…….……spalt-like transcription factor 1

sEPSC .....spontaneous excitatory postsynaptic current

siRNA .....small interfering RNA

SPI1……..……..…….……spleen focus forming virus proviral integration 1

TGF…….transforming growth factor

TLR…….toll-like receptor

TMEM119……...……transmembrane protein 119

TNF…….…… tumor necrosis factor

CHAPTER ONE: INTRODUCTION

Disclaimer: Figures and portions of the text in chapter one were published in Yeh and Ikezu, *Trends in Molecular Medicine* (2019)

1.1 The role of microglia during development and adulthood

Microglia is emerging as a key player in mediating neurodevelopment and maintaining homeostasis in adulthood. Microglia are described as highly dynamic and heterogeneous depending on age and environment. Microglia migrates from the yolk-sac to the central nervous system (CNS) during embryonic development and promotes neuron differentiation and synapse formation. In adulthood, microglia survey the brain for pathogens and clears debris to maintain homeostasis.

1.1.1 Origin and function of microglia, the primary immune cell in the brain

The brain is under constant surveillance by immune cells called microglia to maintain homeostasis throughout development and adulthood. Microglia are distributed throughout the CNS and account for 5-20% of the total glial cell population, depending on region and developmental stage (Ginhoux et al. 2013). Microglia are distinct from other neuronal and glial cells in the CNS as they are derived from the embryonic yolk sac rather than neuronal progenitor cells (Kierdorf et al. 2013). In 1939, Del Rio-Hortega first introduced the term “microglia” to describe ramified cells with migratory and phagocytic activity in the brain that are distinct from neurons, astrocytes, and oligodendrocytes (Rio-Hortega 1939). In humans, microglia-like cells can be detected as three weeks of estimated gestational age and become highly ramified and mature postnatally (Hutchins

et al. 1990; Rezaie et al. 2005). In mice, microglia progenitor cells start migrating from the yolk-sac to the CNS around embryonic day E9.5-10.5 until the formation of the blood-brain barrier at E13.5-14.5 (Kierdorf et al. 2013; Gomez Perdiguero et al. 2015).

Following entry to the brain, microglia undergo a stepwise program of maturation process dependent on the developmental phases of the brain (Matcovitch-Natan et al. 2016). Early prenatal microglia genes expression are enriched with genes associated with cell proliferation and cell cycle, indicating expansion of microglial population. Postnatal early microglia expresses genes related to neural migration, neurogenesis, and cytokine secretion, suggesting a distinct role supporting the production of neural precursor cells, neural maturation, release neurotrophic factors promoting neuronal survival, and synaptic pruning (Ueno et al. 2013; Fujita et al. 2020; Matcovitch-Natan et al. 2016). Mature microglia transition from supporting neurodevelopment to a more homeostatic role mainly involved in surveillance in adulthood. Microglia continue to proliferate and mature until they reach the adult numbers required to maintain homeostasis (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005; Schwarz, Sholar, and Bilbo 2012; Nikodemova et al. 2015). Any disturbances during the critical developmental stage of microglia may alter brain circuitry leading to a pathological state.

Microglia can actively sense changes in the brain by protruding and retracting their processes, expressing many receptors for different environmental cues (Crotti et al., 2016; Nimmerjahn et al., 2005). Microglia express a set of genes associated with sensing endogenous ligands called the ‘sensome’ that enable microglial to detect surrounding changes and adapt to the environment (Hickman et al. 2013). Microglia constantly survey

its environment to detect pathogens or tissue damages in the brain, which is an immune-privileged site protected by the blood-brain barrier (Daneman 2012). Upon brain injury and release of damage-associated molecular patterns in the brain, microglia are activated and migrate toward the damaged area in an ATP-dependent manner and clear debris for the wound healing process (Davalos et al. 2005). In normal physiological conditions, microglial return to homeostatic function. But in disease states, chronic activation of microglia can facilitate neuronal death and exacerbate neurodegeneration.

Microglia can alter their profiles and acquire different states throughout development and adulthood depending on surrounding environmental cues or the brain region. Homeostatic microglial can be identified by increased expression of Fc receptor-like S and scavenger receptor (Fcrls), purinergic receptor P2Y12 (P2ry12), and transmembrane protein 119 (Tmem119). C-type lectin domain family 7 (Clec7a) is enriched in inflammatory and neurodegenerative microglia associated with impaired phagocytosis function (Wendeln et al. 2018; Krasemann et al. 2017). Recent studies have identified subtypes of early postnatal microglia found in the white matter known as axon tract-associated microglia (ATM) or proliferative region-associated microglia (PAM) (Hammond et al. 2019; Q. Li et al. 2019). ATM and PAM are microglia associated with regions of active oligodendrogenesis and play a critical role in mediating myelination (Hammond et al. 2019; Q. Li et al. 2019). Interestingly, Clec7a is enriched in early postnatal PAM with distinct cytokine and chemokine profiles linked to aging microglia (Q. Li et al. 2019; Krasemann et al. 2017; Mrdjen et al. 2018; Safaiyan et al. 2021). Clec7a postnatal microglia populated in white matter regions were associated with

neurogenic niches and had a higher phagocytic capacity, indicating Clec7a involvement in neurogenesis and phagocytosis of newly formed oligodendrocytes (Q. Li et al. 2019). Pharmacological or genetic perturbation of microglia phenotype causes deficits in the oligodendrocyte development and myelination (Shigemoto-Mogami et al. 2014; Hagemeyer et al. 2017; Erblich et al. 2011). Microglia activation by interleukin-1 beta (IL1b) stimulation in neonatal mice leads to hypomyelination, which can be prevented by selective inhibition of activated microglia using gadolinium chloride (Miron et al. 2013). In early postnatal period, microglia-derived neuropilin-1 (Nrp1) supports oligodendrocyte precursor cell proliferation and expansion in the white matter brain region (Sherafat et al. 2021). Most immature and amoeboid microglia in the white matter transiently express Nrp1 (Sherafat et al. 2021). Together, these results show that microglia have a wide range of effects on myelination and supporting oligodendrocytes population. Microglia can phagocytose newly formed oligodendrocytes and support the expansion of oligodendrocyte precursor cells during development.

Recent studies support the idea that microglia maintain their cell by self-renewal in the healthy adult brain (Tay et al. 2018; Askew et al. 2017). Other myeloid cells, such as bone marrow-derived monocytes, only infiltrate the brain due to age-associated neuroinflammation or pathological circumstances such as radiation or brain injury when the blood-brain barrier is compromised (Schulz et al. 2012; Mildner et al. 2007; Minogue et al. 2014; Blau et al. 2012). Recent single-cell RNA-sequencing studies have revealed distinct genes specific to yolk-sac-derived microglia to distinguish from bone marrow-derived myeloid cells (Buttgereit et al. 2016). Spalt-like transcription factor 1 (Sall1) is

identified as the microglia-specific marker to distinguish Sall1+ microglia from Sall1− border-associated macrophages (BAMs) and infiltrating peripheral monocytes (Goldmann et al. 2016; Buttgereit et al. 2016; Mrdjen et al. 2018). However, the functional differences between resident microglia, BAM, and infiltrating peripheral macrophage remain unclear, and how microglia can mediate neuronal development and physiology remain to be determined.

1.1.2 Microglia modulate neural circuitry and synapse development

Microglia play essential roles in supporting the maintenance of homeostatic function in the brain and contributing to innate and adaptive immune responses. Microglia are crucial to normal neurodevelopment. Microglia are the first glial cells in the developing brain that can regulate neurogenesis, myelination, angiogenesis, and synaptic remodeling (Salter and Stevens 2017). During the first two weeks after birth in mice, microglia depletion led to long-lasting behavior deficits associated with behavioral disinhibition and hyperlocomotion (VanRyzin et al. 2016). Temporary microglia depletion by liposomal clodronate resulted in hyper-locomotive, decreased anxiety-like behavior in open field, and elevated plus maze (VanRyzin et al. 2016). Early depletion of microglia also led to perturbations in brain structure with enlarged ventricles and cortical thinning, resembling a degenerative state (VanRyzin et al. 2016). Furthermore, microglial insulin-like growth factor (IGF-1) is critical for preventing layer V cortical neurons cell death during development (Ueno et al. 2013). Transient ablation of microglia led to increased cell deaths of layer V neurons due to a lack of microglia-derived growth factors

IGF1 and chemokine receptor CX3CR1 (Ueno et al. 2013). These findings highlight the essential role of microglia in supporting neuron survival and proper development in a region and age-dependent manner.

During development, microglia shape the neural circuitry by modulating synapse formation and elimination in an activity-dependent manner (Zhan et al. 2014; Stowell et al. 2019). Microglia-neuron communication involves the exchange of cytokines, neurotransmitters, and neuropeptides. Microglia can extend their processes and briefly contact neuronal synapses, contributing to modifying and eliminating unnecessary synaptic structure by various mechanisms, including complement-dependent synapse pruning (Parkhurst et al. 2013; Tremblay, Lowery, and Majewska 2010; Paolicelli et al. 2011). Microglial phenotypes are region-dependent, with differences in density, membrane morphology, and transcriptome profiles (Grabert et al. 2016; De Biase et al. 2017). The region dependency shows that different regions of the brain require distinct microglial functions. In the hippocampus, synapse elimination depends on microglial CX3CR1, as less active synapses with immature spines are eliminated based on neuronal activity (Paolicelli et al. 2011; Y. Zhan et al. 2014). Reduction in neural activity, such as due to whisker trimming or visual deprivation, induces microglial synaptic engulfment and synapse elimination via CX3CR1-CX3CL1 signaling in the barrel cortex (Gunner et al. 2019; Tremblay, Lowery, and Majewska 2010). Many studies have consistently demonstrated microglia contact and synaptic elimination are modulated by experience-dependent neuronal activity (Tremblay, Lowery, and Majewska 2010; Wu et al. 2015). Others have found that microglia can mediate synaptic remodeling via complement

pathways in the lateral geniculate thalamic nucleus or via purinergic P2Y12 in the visual cortex (Schafer et al. 2012; Sipe et al. 2016; Schecter et al. 2017).

Live-cell imaging studies reveal microglia survey the CNS and shape neural circuitry by influencing the number, morphology, and physiology of dendritic spines (Akiyoshi et al. 2018; Tremblay, Lowery, and Majewska 2010; Weinhard et al. 2018). Synaptic pruning by microglia involves immune components, including the complement system, fractalkine/ADAM10, and TREM2-mediated pathways during specific critical periods (Werneburg et al. 2020; Schafer et al. 2012; Filipello et al. 2018). Failure to eliminate synapses can prevent the strengthening of appropriate synaptic connections and inefficient neuronal transmission. In contrast, excessive synapse elimination by over-activation of the complement pathway in microglia is associated with neurodevelopmental diseases such as schizophrenia and spinal muscular atrophy (Vukojicic et al. 2019). Microglia expressing complement receptors can detect and eliminate neuronal synapses marked with complement proteins C1q and C3 (Vukojicic et al. 2019). C1q deficiency led to impaired synapse refinement during early development, which was associated with hyperconnectivity in the motor neurons (Vukojicic et al. 2019). Complement factor C4 overexpression leads to enhanced microglia engulfment of PSD95 and decreased filopodia and immature spine density (Comer et al. 2020). Chemokine receptor CX3CR1 is another critical factor in regulating microglia-neuron communication and synapse connectivity. Knockdown of microglial CX3CR1 leads to a transient reduction in microglial density, impaired synapse pruning causing deficient formation of multisynapse boutons and weakened functional brain connectivity in

adulthood (Y. Zhan et al. 2014). Microglial CX3CR1 deficiency are associated with behavioral deficits including impaired sociability, increased repetitive behavior, and deficits in motor learning, spatial memory, and fear condition with reduced long-term potentiation (Rogers et al. 2011; Y. Zhan et al. 2014).

Another group showed that selective partial phagocytosis of presynaptic boutons and axons, referred to as trogocytosis, facilitates circuit maturation (Weinhard et al. 2018). Microglia use trogocytosis of the axonal compartments independent of the complement pathways. Microglia-induced relocation of spine head filopodia can stabilize synapse formation (Weinhard et al. 2018). Weinhard et al. proposed that spine head filopodia induction may be associated with perturbations in the extracellular by microglial extension or microglial release of chemotactic factors to promote spine formation. Microglia contact at postsynaptic sites induces transient filopodia formation, contributing to the formation of new spine-bouton connections and alter synaptic activity and plasticity (Miyamoto et al. 2016). During synaptogenesis, microglia contact with dendrites can elicit calcium transients upon filopodia formation (Miyamoto et al., 2016). Importantly, microglial activation may be required to induce filopodia formation as treatment with minocycline reduced microglial activation and significantly decreased the filopodia formation rate by microglia contact (Miyamoto et al. 2016). The microglia contact-induced filopodia formation is only observed when the microglia appeared in an activated and amoeboid state during early postnatal P8-10 mice (Miyamoto et al. 2016). Microglia depletion around the postnatal period leads to weakened layer IV synaptic inputs to layer II/III pyramidal neurons, suggesting that amoeboid microglia can facilitate

synapse formation of selective inputs dependent on age and brain region (Miyamoto et al. 2016). Previous studies have revealed that microglia can play an essential role in learning-induced synapse formation and dendritic spine turnover in the adult brain (Parkhurst et al. 2013). Parkhurst et al. showed that selective ablation of microglia or microglial BDNF decreased spine formation associated with different learning tasks (2013). In the hippocampus region with the highest expression of BDNF, neuronal BDNF prevents microglia from phagocytosing mossy fiber synapses, suggesting that neuron-microglia communication is necessary for synapse maintenance (Onodera et al. 2021). Selective ablation of microglial BDNF increases the production of newborn neurons (Harley et al. 2021). In addition to regulating neurogenesis, microglial BDNF seems critical in response to LPS, as well as reduced microglial self-renewal capacity (Harley et al. 2021).

Microglia regulate neurogenesis by a range of mechanisms, including providing trophic support or modulating neurogenic niche proliferation and survival (Sierra et al. 2010; Shigemoto-Mogami et al. 2014; Antony et al. 2011; Ueno et al. 2013, Diaz-Aparicio et al. 2020). Chronic deficiency of phagocytosis due to impaired purinergic P2RY12 and tyrosine kinases MerTK/Axl pathway signaling resulted in dysregulated neurogenesis (Diaz-Aparicio et al. 2020). In hippocampal neurogenic niches, microglia control the proliferation and differentiation of neural stem cells and phagocytose newborn cells that undergo apoptosis (Gebara et al. 2013; Sierra et al. 2010, Vukovic et al. 2012; Rodríguez-Iglesias, Sierra, and Valero 2019). Microglia can secrete metabolites, miRNAs, and extracellular vesicles, altering neurogenesis (Rodríguez-Iglesias, Sierra,

and Valero 2019). There are contradictory results regarding whether inflammation promotes or inhibits adult neurogenesis. Some studies show that pro-inflammatory cytokines, including IL1b, IL-6, and TNFa, inhibit adult neurogenesis (Breton and Mao-Draayer 2011; Ekdahl et al. 2003; Monje, Toda, and Palmer 2003). However, a more recent study concluded that inflammation due to LPS stimulation did not inhibit adult neurogenesis and that phagocytotic microglia modulate neurogenesis by restricting differentiation of neural-committed cells (Diaz-Aparicio et al. 2020).

More recent studies demonstrate the active role of microglia in mediating neuronal activity. Neuronal activation can alter microglia-neuron interaction through activity-induced synaptic release of ATP (Eyo et al. 2014; Badimon et al. 2020). ATP can act as a local chemoattractant that recruits microglial protrusions to activated synapses or neuronal somas (Badimon et al. 2020; Cserép et al. 2020). Microglia can sense neuronal-derived ATP by purinergic receptor P2RY12 and provide negative feedback to inhibit excessive neuronal activity (Badimon et al. 2020). P2Y12R knockout mice displayed increased excitability in ventral hippocampus CA1 neurons, suggesting a key role of microglia in suppressing neuronal activity (Peng et al. 2019). In contrast, another group has shown that direct microglial contact with a synapse leads to local Ca2+ influx in dendritic spines, and an increase in back-propagating action potentials along the dendrite, suggesting increased excitability of the neuron (Akiyoshi et al. 2018). Interestingly, Akiyoshi et al. found that microglia ablation reduces synchronous firing of layer II/III neurons suggesting microglia helps to synchronize local populations of neurons in the primary motor cortex (2018). This discrepancy of microglial effect on neuronal

synchrony may be explained by region differences in microglial profile or composition of neuronal populations or basal neuronal activity due to different inputs. Further studies may reveal how microglia affects neuronal synchrony depending on neural circuits in an activity-dependent manner in vivo of awake animals.

These findings highlight that microglia may support neural circuitry development *via* multiple pathways. Therefore, abnormal microglia activity due to inflammation or insult can contribute to behavior and neuronal deficits in neurodevelopmental and neurodegenerative disorders.

1.2. Factors that can affect microglia function and transcriptome

Microglia are constantly changing and adapting to the microenvironment of the brain. The microglia phenotype can be influenced by intrinsic factors, such as sex and age, and environmental cues, such as protein aggregates, stress signals, diet, or cytokines secreted from gut microbiota (Erny et al. 2015). Under pathological conditions, cell debris or stress signals in the brain can lead to microglial transcriptomic and morphological changes associated with reactive microglia (Crotti and Ransohoff 2016). In this section, both genetic and environmental factors that can influence the microglia condition and transcriptome will be discussed (**Figure 1**).

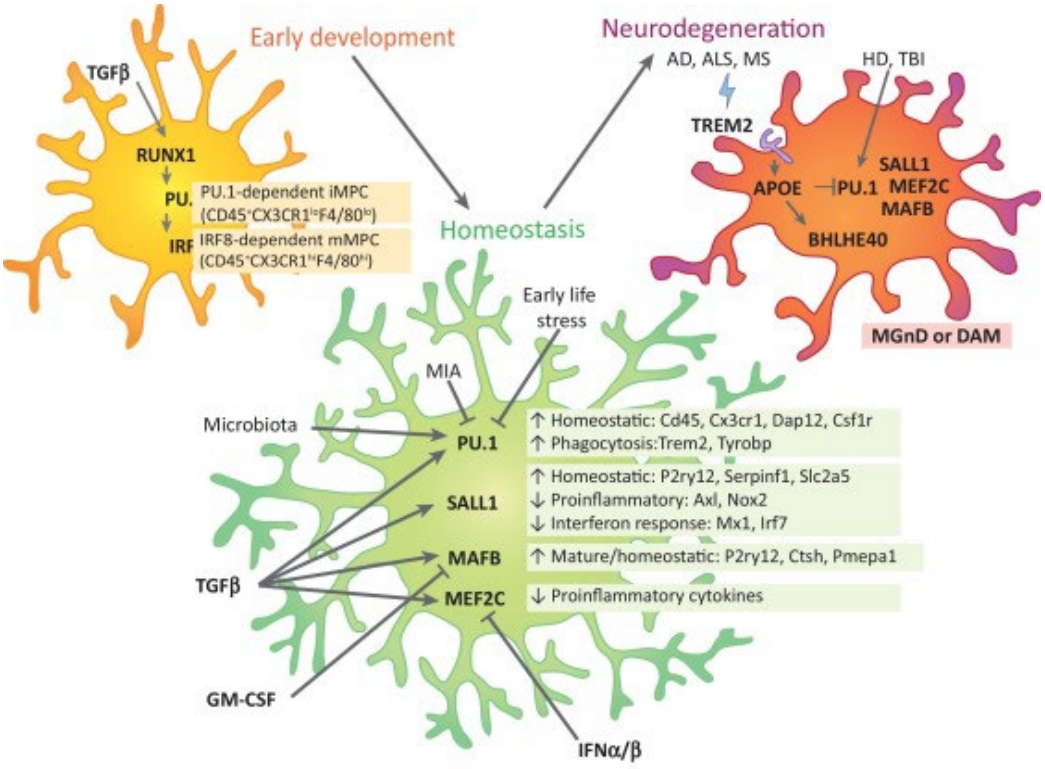

Figure 1. Key microglial transcription factors regulating microglial transcriptome and functions during early development, homeostasis, and neurodegeneration.

In immature microglia (yellow), the transcription factors Runt-related transcription factor 1 (RUNX1), SPI/PU.1, and interferon regulatory factor 8 (IRF8) are highly expressed and promote differentiation of microglia to establish microglial cell lineage. In homeostatic microglia (green), PU.1, Spalt like transcription factor 1 (SALL1), V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB), and myocyte enhancer factor 2c (MEF2c) promote expression of microglial homeostatic genes. Neurodegenerative microglia (red) are characterized by increased apolipoprotein E (APOE) pathway signaling by activating triggering receptor expressed on myeloid cells 2 pathways (TREM2; purple). Alzheimer's disease (AD); amyotrophic lateral sclerosis (ALS), granulocyte-macrophage colony-stimulating factor (GM-CSF); type I interferon (IFN); immature myeloid precursor cell (iMPC); maternal immune activation (MIA); mature myeloid precursor cell (mMPC); multiple sclerosis (MS); Huntington's disease (HD); traumatic brain injury (TB); transforming growth factor beta (TGFβ). Figure by H. Yeh and published in Yeh and Ikezu. *Trends in Molecular Medicine* (2019).

1.2.1 Genetic factors that can influence microglia

Microglial transcriptomic profile and functions are regulated by transcription factors including Spi1/PU.1, Sall1, Mafb, and Mef2c (Yeh and Ikezu 2019). PU.1 is the most abundantly expressed transcription factor in microglia and promotes microglia differentiation and identity maintenance (Gosselin et al. 2017). PU.1 is upregulated in erythromyeloid progenitors (EMPs) and expressed throughout development and adulthood, suggesting the necessity of PU.1 in addition to IRF8 for microglial development and maintenance (Kierdorf et al. 2013). About 63% of the microglial genes for sensing endogenous ligands and microbes, termed microglia sensome, are PU.1 targets, suggesting that PU.1 regulates microglial-specific functions (Satoh et al. 2014). Reduced microglial expression of PU.1 can lead to decreased ramifications, phagocytic functional changes and reduced expression of MHC class II genes, indicative of altered function in antigen presentation and proinflammatory response (A. M. Smith et al. 2013). PU.1-deficient mice show immune-related abnormalities and reduced expression of colony-stimulating factor receptors (Csf1r, Csf2r, and Csf3r), which are critical receptors for maintaining immune and microglial cell survival (Beers et al. 2006; Rosenbauer et al. 2004).

Microglia can phagocytose and remove apoptotic cells and debris in a brain region-dependent manner. In the adult brain, microglial clearance activity is diminished in regions with reduced neuronal deaths (Ayata et al. 2018). Epigenetic modification by H3K27me3 can suppress microglial phagocytosis via reducing expression of phagocytosis-associated genes. In comparison to striatal microglia, cerebellar microglia

display a more phagocytic and amoeboid phenotype resembling immature microglia and MGnD/DAM but lack enrichment of proinflammatory genes (Ayata et al. 2018). These studies demonstrate that microglial profile and phagocytosis capacity can change based on epigenetic changes in the brain.

1.3 The impact of immune dysfunction during development

Epidemiological studies found a significant association between maternal infection and neurodevelopment disorders, including autism spectrum disorder (ASD), anxiety, bipolar disorder, schizophrenia, and major depressive disorder (Brown and Patterson 2011; Estes and McAllister 2016; Parboosing et al. 2013). Prenatal exposure to infectious stimuli such as influenza, rubella, herpes complex, measles, and bacteria pathogens leads to a higher incidence of psychiatric disorders, including schizophrenia and ASD (Buka et al. 2001; Brown et al. 2001; Sørensen et al. 2009). More specifically, admission to hospital due to maternal infection in the first trimester and maternal bacterial infection in the second trimester significantly increased the risk for developing ASD in the offspring (Atladóttir et al. 2010).

Exposure to upregulation of cytokines by MIA is an environmental risk factor for neurodevelopment and neuropsychiatric disorder. Proinflammatory cytokines IL6, IL1β, and TNFα are elevated in serum samples of patients with schizophrenia or ASD (Boerrigter et al. 2017; Eftekharian et al. 2018; Saghazadeh et al. 2019). Maternal inflammation leads to behavioral deficits observed in neurodevelopmental disorders that can be corrected by treatment with minocycline (Mattei et al. 2017; Xia et al. 2020). This

section discusses how immune activation can disrupt normal brain development and affect behavior outcomes.

1.3.1 Environmental factors leading to inflammation and alteration in microglia profile

Local cues or factors secreted by neurons or glial cells can affect microglial transcriptome and function in the brain. When there are changes in CNS developmental periods or integrity, microglia react accordingly. Early perinatal microglia resemble amoeboid morphology and increased production and secretion of cytokine TGFB1 promotes homeostatic microglia and has been shown to shape the chromatin landscape and influence the response and phenotype of microglia (Butovsky et al. 2014; Cohen et al. 2014). The impact of stress is circuitry-specific and regimen-dependent. Stress increases dorsal medial prefrontal cortex spine elimination without affecting spine formation (Liu et al. 2021). Stress reduces mPFC microglia branching complexity and contacts with dendritic spines, and enhances synapse elimination (Liu et al. 2021).

The gut microbiome has emerged as a critical factor for mediating microglial transcriptome and physiology. Gut microbiota can induce transcriptomic changes in microglia associated with microglial differentiation and identity. Gut symbiosis promotes the maintenance of homeostatic microglia, while gut dysbiosis under germ-free (GF) conditions impairs microglia differentiation, maturation, and functions (Erny et al. 2015). RNA-seq analysis reveals GF conditions increase microglial expression of PU.1 and survival factor CSF1R, suggesting microbiota can contribute to microglia profile (Erny et

al. 2015). Thion et al. demonstrated that microglial transcriptomics depend on sex, age, and presence of microbiota, with female embryonic microglia and male adult microglia more resilient to microbiota depletion (Thion et al. 2019).

Maternal immune activation (MIA) is found to be another factor mediating microglial transcriptome and function (Reisinger et al. 2015). Early maternal inflammation accelerates microglial maturation, dysregulates gene expression associated with inflammation, cell migration, and phagocytosis (Matcovitch-Natan et al. 2016; Mattei et al. 2017). MIA reduces PU.1 expression and its target genes in microglia, including microglial sensome genes, which can be restored by anti-inflammatory minocycline treatment (Mattei et al. 2017). MIA microglia transcriptome has significant overlap with microglia isolated from APP/PS1 mice, suggesting that MIA and neurodegenerative disorder may share some molecular mechanisms leading to dysregulated microglial function (Mattei et al. 2017). More recent studies have found a connection between microbiota and MIA-induced behavioral deficits (Hsiao et al. 2013; Yim et al. 2017). One study showed that MIA could induce dysregulation of the MIA offspring's microbiome and behavior symptom severity (Hsiao et al. 2013; Yim et al. 2017). MIA offspring had altered gut microbiota composition, and oral treatment with human commensal *Bacteroides fragilis* ameliorated MIA phenotypes, including behavioral abnormalities (Hsiao et al. 2013). Another study suggests that MIA-induced deficits require a specific intestinal bacteria *SFB* that promote T helper 17 cell differentiation and upregulation of IL-17 during the gestational period (Kim et al. 2017).

These results revealed that microbiota may play a critical role in the gut-brain axis and alter early neurodevelopment.

An emerging environmental issue is air pollution and its impact on brain development. Exposure to air pollution can lead to chronic inflammation and impair brain function. Mice chronically exposed to air pollution particulate have increased reactive microglia and local deposition of complement proteins C5/ C5α and C5α receptor CD88 in the corpus callosum (Babadjouni et al. 2018). Gestational exposure to diesel exhaust particles increases inflammatory cytokine secretion and alters microglial morphology, consistent with activated or delayed maturation in the embryonic brain of male mice (Bolton et al. 2017).

Another environmental factor influencing maternal inflammation and microglia phenotype is the dietary intake of polyunsaturated fatty acids (PUFA) during pregnancy. Deficient maternal intake of omega-3 PUFA during neurodevelopment results in vision and cognition impairment in mice (Weiser, Butt, and Mohajeri 2016; Delpech et al. 2015). Excessive consumption of omega-6 PUFA during pregnancy leads to social deficits and impaired cognition in offspring, as observed in the MIA mouse model (Jones et al. 2013). Supplementation with omega-3 PUFA or docosahexaenoic acid reduce inflammation and prevented the onset of behavior deficits in MIA offspring (Li et al. 2015; Weiser et al. 2016). This anti-inflammatory effect of omega 3 PUFAs could be acting on reducing microglial activation. Maternal omega-3 PUFA deficiency alters microglia fatty acid composition, increases microglia-mediated synaptic refinement in a complement-dependent manner, and results in the upregulation of genes associated with

immune and inflammatory pathways (Madore et al. 2020). Deficient maternal omega-3 PUFA intake also includes behavior deficits in working memory and reduced dendritic length and spine density (Madore et al. 2020). Therefore, dietary fatty acids can mediate microglia function and alter brain circuitry during early development.

In postnatal mice, early life stress can also affect microglia development and neuronal circuitry. Early life stress caused by early maternal separation can activate microglial cells and reduce microglial PU.1 transcriptional activity (Delpech et al. 2016). On the other hand, environmental enrichment can also have an impact on microglial density. Enrichment housing of mice led to an increased microglia density and branching complexity (Xu et al. 2016). Previous studies demonstrate enriched environment can alleviate the progression or severity of specific neurological diseases such as Alzheimer's disease or depression (Williamson et al. 2011; H. Xu et al. 2016; Chabry et al. 2015). The increased density and hyper-ramification may reflect a neuroprotective state rather than a pro-inflammatory state based on the normalization of inflammatory markers in the brain induced by aging or amyloid treatment (Xu et al. 2016). Microglia can alter phenotype based on local surrounding cues in the brain and external cues, including microbiota, maternal inflammation, dietary intake of fatty acid, environmental enrichment.

Studies have also shown that the Wnt signaling pathway can mediate microglia reactivity and contribute to neuroinflammation in neurodevelopment and neurodegenerative disorders (Yang and Zhang 2020). Wnt is involved in cell survival, proliferation, and differentiation of neuronal precursors (Kalani et al. 2008). β-catenin accumulates in activated microglia in Alzheimer's disease patients depending on age and

disease progression (Halleskog et al. 2011). Dysregulation of the canonical Wnt/β-catenin pathway leads to a more pro-inflammatory phenotype with increased expression CD68, CD33, and CD11b (Halleskog et al. 2011; Halleskog and Schulte 2013; Mrdjen et al. 2018). Direct IL1-b treatment to activate microglia results in downregulation of the canonical Wnt pathway, suggesting a critical role of Wnt in mediating inflammatory response (Van Steenwinckel et al. 2019). Pharmacological inhibition of the Wnt/β-catenin pathway promotes pro-inflammatory microglial phenotype i*n vitro* and *in vivo* (Van Steenwinckel et al. 2019). Wnt/β-catenin agonist therapy reducing proinflammatory microglia improves neuropathological and functional outcomes in a mouse model of white matter injury and chronic cerebral ischemia (Van Steenwinckel et al. 2019; Song et al. 2019). Wnt3a can activate microglia by evoking cytokine release and altering phagocytotic activity (Halleskog et al. 2011). Wnt3a treatment activating Wnt/β-catenin pathway rescues microglia survival in Triggering Receptor Expressed on Myeloid cells 2 (Trem2)-deficient mice, suggesting modulating Wnt pathway may improve microglial survival and microgliosis associated with Alzheimer's disease with TREM2 dysfunction (Zheng et al. 2017). However, how Wnt pathway dysfunction affects microglial function and microglia-neuron interactions remain to be determined by additional studies.

1.3.2 Inflammation and risk of neurodevelopment disorder

Genome-wide association studies (GWAS) have shown that the region encoding major histocompatibility (MHC) and innate immune genes with the most significant association with schizophrenia (Stefansson et al. 2009; Shi et al. 2009). Complement

component 4A (C4A) is one of the immune genes located in the MHC locus and highly associated schizophrenia, and structural variants that increase the expression of C4A lead to increased susceptibility for schizophrenia (Sekar et al. 2016). The complement signaling cascade is part of the innate immune system that identifies and marks pathogens and apoptotic cells for phagocytosis by macrophages (Veerhuis, Nielsen, and Tenner 2011). In contrast, GWAS for ASD have not uncovered any immune-related genes significantly associated with increased susceptibility to ASD (Wang et al. 2009; Weiss et al. 2009; Anney et al. 2010). However, genetic studies revealed that genomic variations in the MHC region are also significantly associated with increased susceptibility to ASD (Lee et al. 2006; Torres et al. 2006; Guerini et al. 2011). These GWAS and genetic studies collectively suggest that MHC and immune response genes may contribute to the emergence of ASD and schizophrenia patients. However, the exact roles of these genes and how it impacts microglial function may need to be addressed in future studies.

Gene expression changes found in human postmortem brain tissue can offer insights into cell type-specific gene expression and pathway changes due to specific psychiatric disorders. Transcriptome study of postmortem brain tissue show synaptic, oligodendrocyte, and energy metabolism-related disturbances in schizophrenia patients (Faludi and Mirnics 2011). On the other hand, transcriptomic analyses reveal upregulation of genes associated with activated microglia in the post-mortem cortex of ASD patients (Voineagu et al. 2011; Gupta et al. 2014). Although immune dysregulation is implicated in ASD patients, it remains unclear if underlying genetic variants cause

immune profile changes or whether it represents an immune response to neuronal abnormality.

Animal models allow the opportunity to study the molecular mechanisms driving neuropathology associated with MIA. Pathological changes due to MIA have been reported in both neurons and glial cells, including microglia, and associated with deficits in cell migration, proliferation, and maturation during early development (Yim et al. 2017; Soumiya, Fukumitsu, and Furukawa 2011; Canales et al. 2021). MIA offspring exposed to maternal inflammation due to synthetic viral mimic polyinosinic:polycytidylic acid [poly(I:C)] or bacterial mimic lipopolysaccharide (LPS) shows behavior deficits recapitulating neuropathies and aberrant behaviors observed in those with neurodevelopment disorder (Machado et al. 2015; Depino 2015; O'Loughlin et al. 2017; Malkova et al. 2012).

Following the immune challenge, acute transcriptomic changes in the fetal brain of MIA offspring show significant overlap with changes in the cortices of children with ASD (Lombardo et al. 2018). Acute response to MIA includes dysregulated transcriptomic changes associated with hypoxia, immune signaling, metabolic and angiogenesis pathways. Maternal inflammation leads to an increased number of putative astrocytes and oligodendrocytes typically born at later stages of corticogenesis, supporting findings of accelerated cell development (Canales et al., 2021). More lasting effects of MIA on the brain transcriptome include altered hypoxia, cellular metabolism, and neuroimmune gene pathway changes in postnatal development (Canales et al. 2021). MIA offspring demonstrate altered cortical thickness and cortical dysplasia, or even

stereotypic dysplasia impacting lamination located in the somatosensory cortex (Smith et al. 2007; Soumiya, Fukumitsu, and Furukawa 2011; Choi et al. 2016; Yim et al. 2017). These provide evidence that the MIA rodent model can be a valuable model for studying the molecular mechanisms of immune-associated psychiatric disorders.

MIA results in increased inflammation in the fetus and fetal brain, with elevated levels of pro-inflammatory cytokines including TNFα, interferon-γ (IFNγ), and IL-1b and IL-6 (Goines et al. 2011; Ballendine et al. 2015; Hsiao et al. 2012; Meyer et al. 2006; Smith et al. 2007). IL-6 is considered the primary mediator of maternal inflammation, and IL-6 administration to pregnant dams at embryonic day 12 (E12.5) replicates the effects of MIA induction by poly(I:C) (Smith et al. 2007). IL-6 treatment also altered the gene expression of fetal cortex and behavior outcomes comparable to autism and schizophrenia patients (Smith et al. 2007). MIA-behavioral deficits correspond to symptoms observed in psychiatric patients, including reduced pre-pulse inhibition, latent inhibition, social interactions, and increased anxiety in the open-field test (Smith et al. 2007). Hence, immune challenges during early prenatal development can lead to cytokine dysregulation and behavioral deficits.

1.3.3 Microglia dysfunction contributes to the pathogenesis of neuropsychiatric disorders

Schizophrenia and autism spectrum disorder share a subset of risk factors and symptoms, including social interaction and communication deficits. Post-mortem studies of brains from patients with schizophrenia and ASD have identified microglia anomalies

and altered inflammatory processes (Vargas et al. 2005; Morgan et al. 2010; Trépanier et al. 2016; van Kesteren et al. 2017). Synaptic density is reduced in postmortem cortical brain tissue of schizophrenia patients, suggesting excessive synapse elimination by microglia (Petanjek et al. 2011; Glausier and Lewis 2013; Glantz and Lewis 2000; Konopaske et al. 2014). In a human schizophrenia patient iPSC derived-microglia-like cells and neuron co-culture, synapse engulfment depends on microglial disease status rather than neuronal phenotype, but neuronal and microglia factors both contribute to excessive synaptosome engulfment (Sellgren et al. 2019). C4 variants associated with schizophrenia patients caused an excessive neuronal complement deposition mediated by C4a to increase microglia synapse engulfment (Sellgren et al. 2019). Minocycline treatment reduced synapse engulfment and increased spine density *in vitro* (Sellgren et al. 2019). Notably, treatment of minocycline during adolescence is significantly associated with a reduced risk of psychosis incidents (Sellgren et al. 2019). Therefore, understanding the molecular mechanisms of microglia biology may further the development of therapeutics for schizophrenia.

Genetic studies show dysregulation of protein synthesis in ASD patients. Upregulated protein synthesis leads to synaptic changes associated with social deficits in male mice (Hutsler and Zhang 2010; Xu et al. 2020). On the other hand, fragile X syndrome is known to be caused by reduced expression of fragile X mental retardation protein (FMRP) and over-activation of the mTORC1-eIF4E pathway involved in protein synthesis (Hoeffer et al. 2012). In mice, the elevation of mRNA translation in microglia by overexpression of eIF4E is sufficient to cause autistic-like phenotypes by altering

microglia-neuron interactions (Xu et al. 2020). Increased spine density, postsynaptic neuroligins, and excitation/inhibition imbalance lead to social interaction, repetitive behavior, and cognitive deficits in male mice (Xu et al. 2020). Furthermore, transcriptome analysis reveals elevated protein synthesis in microglia results in impaired microglia motility and reduced microglial engulfment of synapses (Xu et al. 2020).

However, there have been no genetic studies identifying ASD-associated causal genetic variants in microglia. ASD-associated mutations in ubiquitously expressed genes can contribute to dysregulated development through acting on microglia (Xu et al. 2020). Thus, there is a need to address how perturbations to microglia-neuron interactions can affect brain development and neuronal function. Here, we investigate how maternal immune activation can mediate neuronal physiology and behavioral outcome through altering microglial phenotype and function. Maternal inflammation has been implicated in altering brain circuitry and behavior, but the role of microglia remains elusive. We further address how microglial Wnt gene WNT5a can change neuron development and function.

CHAPTER TWO: MATERIALS AND METHODS

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2.1 Animal care and microglia depletion/repopulation

All animal procedures were performed according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Boston University Institutional Animal Care and Use Committee. C57BL6/J mice (Jackson Laboratory) mice were housed in 12 h light / 12 h dark schedule with access to chow ad libitum. Females were combined with males for timed mating, and vaginal plugs are checked the following morning. Pregnancy was determined by the presence of a vaginal plug-in combination with more than 2 g of weight gain from E0 to E9.5. For MIA induction, female mice were injected intraperitoneally (IP) with 20 mg/kg polyinosinic-polycytidylic acid potassium salt dissolved with 0.9% sodium chloride on embryonic day 9.5 (E9.5) (Santa Cruz, sc-202767) (Garbett et al. 2012; W.-Y. Li et al. 2014; Garay et al. 2013; Malkova et al. 2012). Pregnant female mice were housed individually after separation from the male mouse. All offspring were weaned at P21 and group-housed with 2-5 same-sex littermates per cage.

Microglia depletion and subsequent microglial repopulation were completed by the administration of a colony-stimulating factor 1 receptor (CSF1R) inhibitor PLX5622 (Plexxikon, Inc.), which was incorporated into the AIN-76A standard chow by Research Diets, Inc., from P21-42 for microglia repopulation group (MG-REP group) (1200 p.p.m.

delivering daily doses of 168 mg/kg)(Elmore et al. 2014; Asai et al. 2015). The control treatment groups (CTRL) were fed with the standard chow AIN-76A from P21–68.

For labeling of proliferating cells, thymidine analog 5-Bromo-2′-deoxyuridine (BrdU) was repetitively injected from P1 to P20 in control (CTRL) and microglia repopulation (MG-REP) female offspring before PLX administration. Following microglia depletion and withdrawal of PLX-containing chow, 5-Ethynyl-2′-deoxyuridine (EdU) was repetitively injected from P43 to P59 to label the repopulating microglia after microglial depletion (Salic and Mitchison 2008; Askew et al. 2017; Huang et al. 2018).

2.2 Behavior testing

All behavioral tests were performed in a dedicated behavior testing room within the mouse vivarium between 9 a.m. and 6 p.m during the light cycle. The mice were habituated in the testing room for at least 1 h prior to behavior assays. A white noise machine was used during the repetitive behavior test. The lighting, humidity, and room temperature were kept as constant as possible in the testing room. Behavioral recordings were manually analyzed by experimenters blinded to treatment groups.

Three-chamber social interaction test

The social interaction test was based on previous studies (Nadler et al. 2004; Buffington et al. 2016). Boston University Scientific Instrument Facility manufactured an opaque plexiglass three-chambered apparatus. Each mouse started in the middle chamber with wall dividers for 5 min habituation. Then, a novel male mouse similar in age and weight (within 5 g) was placed in the wired cylinder on one side. An identical empty

wired cage was placed on the opposing side chamber and marked as the “empty tube”. The chamber wall dividers were removed, and the test mouse was then allowed to explore freely for 10 min while being video recorded. After completion of behavior assays, all mice were removed, and the apparatus was cleaned with 20% ethanol. The time spent in each chamber and the number of entries into each chamber were video-tracked using Ethovision XT14 software.

Self-grooming test

The self-grooming test was performed as described (Malkova et al. 2012). Briefly, mice were placed individually in 6.5 cm diameter × 12 cm tall clear glass beaker covered by a metal mesh top. Following 10 min habituation period in the beaker, the mice were video-recorded for 10 min. Glass beakers were only used once per day and cleaned with water and soap. Video recordings were analyzed by experimenters blinded to treatment groups. Self-grooming duration was the sum of time each mouse spent grooming their fur with their mouth or paws.

2.3 Isolation of microglia and RNA extraction

According to the manufacturer's protocol, mouse microglia were isolated from the offspring of saline or poly(I:C)-treated pregnant dams using CD11B MicroBeads (Miltenyi Biotec)(Harms and Tansey 2013). CD11B+ microglia cells were homogenized and lysed in Qiazol (Qiagen) and snap-frozen at stored −80 °C. Total mRNA was extracted using the miRNeasy kit accordingly manufacturer's protocol (Qiagen). mRNA

concentration and RNA quality were confirmed with a minimum RIN score of 8.5 by Bioanalyzer 2100 (Agilent Technologies).

2.4 RNA-sequencing library preparation and analysis

Truseq (Illumina) non-stranded cDNA libraries was prepared from poly(A)-enriched mRNA. mRNA-sequencing was performed using Illumina HiSeq2000 at the Massachusetts Institute of Technology (MIT) MicroBio Center using 40-base pair single-end reads, with a minimum of 20 million reads per sample. The Bioconductor Rsubread package in R was used to align reads to the mm10 *Mus musculus* reference genome and obtained feature counts (Liao, Smyth, and Shi 2013). To alignment, the Rsubread function "Align" was used. BAM files for generated for each sample and read summarization using an annotation file (.gtf) from https://genome.ucsc.edu, with the Rsubread function "featureCounts," to assign reads to genomic target features. The BatchQC pipeline was used to check for batch effect (Manimaran et al. 2016). Litter and gender were assessed as potential confounding factors. Principal component analysis was performed using BatchQC. Comparison analyses were performed using EdgeR (Robinson, McCarthy, and Smyth 2010). A generalized linear model with Quasi Likelihood F testing (glmQLFit) was used for paired and group comparisons. Feature count data were grouped, normalized, and filtered to obtain counts per million (CPM) of 2 or above in at least three samples (out of 30 total replicates) with a total number of 14,225 genes. For group comparisons, a model matrix was designed for examining MIA [poly(I:C)] and drug treatment (MG-REP), with a threshold of $p < 0.05$.

ComplexHeatmaps R package and Elbow method were used to perform K-means clustering (k = 5) and generate heatmaps of clustered genes (IM module: 4681 transcripts, MIA-IM module: 2816 transcripts, JM module:2318 transcripts, AM module: 3276 transcripts, REP-AM module: 1134 transcripts). Results with p-values and expression fold changes (MIA versus Saline) were uploaded for IPA (Qiagen) core analyses. The threshold for upstream regulators of biological pathways was $p < 0.01$ in at least one of the four or five comparison groups. Volcano plots were generated by using Multiplot Studio (GenePattern Archive). Venn diagrams were created using Venny 2.1 (BioinfoGP, http://bioinfogp.cnb.csic.es/tools/venny/).

Assessment of microglia purity

Expression of cell-type specific markers, based on Barres dataset (Zhang et al. 2014) of mouse neurons, astrocytes, oligodendrocytes, endothelial cells, and microglia, were cross-examined with our dataset. The following genes: Dcx, Npas4, Npy, Reln, Tubb3 (neurons), Mag, Mbp, Mog, Ppapdc1a, Sox10 (oligodendrocytes), Aqp4, Gfap, Gdpd2, Plcd4, Slc7a2, (astrocytes), Emcn, Ocln, Pecam1, Slc16a4, Tek (endothelial cells), C1qa, Csf1r, Cx3cr1, Fcrls, Hexb (microglia) were used to assess CD11B+ microglia isolation purity. The percentages of each cell type marker were calculated and normalized to the total transcripts per million (TPM).

Gene ontology (GO) biological functions and pathway analysis

We used the DAVID (version 6.8) to assess the enrichment for gene ontology terms (GO Direct_BP; biological process). Genes enrichment was analyzed and adjusted for Bonferroni, Benjamini, and FDR correction for multiple comparisons and visualized

using Prism 6.0 (GraphPad). AM module genes with an expression ratio <1 in MIA versus Saline were sorted for expression ratio in MIA+CTRL versus MIA+MG-REP. The final lists of genes that are decreased in MIA+CTRL versus Saline+CTRL and then increased in MIA + MG-REP versus MIA (or vice-versa) was input to DAVID, and the top ten GO terms (biological process) were visualized, with the background list of 14,225 microglial expressed genes for all DAVID analyses. Raw and FDR-corrected (method: Bonferroni) p-values were determined for significance.

2.5 ELISA validation

Protein was extracted from 1–2 × 10E6 acutely isolated microglial from E17 or P60 mouse brain using TENT buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Cell supernatant was collected after centrifugation at 20,000 × g for 30 min at 4 °C and total protein concentration was determined by BCA assay kit (Pierce/Thermo Fisher). For the detection of NTRK2 (TrkB), PTN, and NTN1, the following commercial ELISA kits were used at 30 µg of protein per reaction.: Mouse PTN (Biomatik Corporation, EKU06717), NTRK2/TrkB (Boster Biological Technology, EK0849), NTN1 (LifeSpan Biosciences, LS-F5882). For comparing protein levels of CTNND2, NCAM2, and WNT5A, custom ELISA kits were developed according to the manufacturer's instruction using anti-CTNND2 antibody (0.3 µg/well, Santa Cruz Biotechnology, SC-81793, mouse monoclonal 40.1), biotinylated anti-CTNND2 antibody (rabbit, 1 µg/ml, Abcam, EPR17628), anti-NCAM2 antibody (goat polyclonal, Acris Antibodies GmbH, AP32136PU-N), biotinylated anti-NCAM2 (goat polyclonal antibody,

0.3 μg/ml, Pierce/Thermo Scientific, 90407), anti-WNT5A antibody (goat polyclonal, 0.3 μg/well, R&D Systems, AF645), and biotinylated anti-WNT5A antibody (1 μg/ml, Pierce Antibody Biotinylation Kit). For standards, CTNND2 protein (Abnova, H00001501-Q01), NCAM2 polypeptide (Everest Biotech, EBP06991), and WNT5A protein (R&D Systems, 645-WN-010) were used. Protein concentration was determined by optical density reading at 450 nm by a microplate reader (BioTek Instruments).

2.6 *In situ* hybridization and immunofluorescence of microglia

In vitro transcription of cRNA probes against mouse Ncam2 3′ UTR, Wnt5a 3′ UTR, Ntn1 3′ UTR, Ctnnd2 3′ UTR, and scramble under the control of the T7 promoter (taatacgactcactataggcg) were synthesized as double-stranded DNA fragment. Digoxigenin (DIG)-labeled cRNA riboprobes were synthesized using DIG labeling mix (1 277 073, Roche Diagnostics GmbH) and Riboprobe In Vitro Transcription Systems (P1420, Promega) and purified using ProbeQuant G-50 microcolumns (GE28-9034, GE Healthcare). *in situ* hybridization was performed for detecting each mRNA probe on brain sections using anti-DIG-POD Fab fragments (11 207 733 910, Roche) and the TSA Plus Cy3 fluorescence system (cat. no. NEL745, Perkin Elmer) as previously described (Asai et al. 2015). After the *in situ* hybridization, the sections were subjected to immunofluorescence procedure as described below to detect microglial IBA1 protein expression.

2.7 Whole-cell patch-clamp recording

Preparation of brain slices for recording and filling

Acute mouse brain slices were harvested and prepared for whole-cell patch-clamp recordings, as previously described (Crimins et al. 2011). Mice were anesthetized with isoflurane prior to decapitation. Each brain was placed into ice-cold oxygenated Ringer's solution for sectioning into 300 μm coronal thick slices using a vibrating microtome. The slices were transferred to room temperature Ringer's solution for at least an hour before recording. For patch-clamp recording, each brain section was transferred to a submersion-type recording chamber affixed to the stage of an IR-DIC Microscope (Micro Video Instruments) and was perfused with oxygenated Ringer's solution. Layer V pyramidal neurons were identified by their distance from the pial surface of the cortex (400–700 μm). Glass electrodes were created from borosilicate glass with a Flaming and Brown micropipette puller (Model P-87, Sutter Instruments) and filled with a potassium methanesulfonate internal solution (Crimins et al. 2011).

Physiological inclusion criteria

Neurons were visually identified as layer V pyramidal neurons and were required to have stable access, low noise, and a resting membrane potential below −55 mV for inclusion in this study. Layer V neurons were classified into regular spiking (RS) or intrinsically bursting (IB) types based on action potential firing patterns: the first inter-spike interval (ISI) was compared with the subsequent ISI in the spike train. RS neurons had an equal first ISI compared with second ISI. IB neurons had a shorter first ISI compared with subsequent ISIs (Yang, Seamans, and Gorelova 1996).

Physiological analysis

PatchMaster acquisition software and EPC-9 or EPC-10 patch-clamp amplifiers (HEKA Elektronik) were used to acquire electrophysiological data. FitMaster Analysis Software (HEKA Elektronik) was used to analyze passive and active membrane properties. The passive membrane properties, resting membrane potential (Vr), input resistance (Rn), and the membrane time constants (Tau) were recorded under current-clamp conditions. Vr was measured as the voltage present when the current injection was zero. Rn and Tau were measured by injecting 20 mV steps starting at −160 mV. Tau was defined by fitting the membrane response to a small current injection (−20 or −40 mV) to a single exponential function. Rn was assessed as the slope of a best-fit line through the V–I plot. Single action potential spike properties, consisting of threshold, amplitude (amp), rise, decay, and duration, were measured from the first single spike recorded on the 200 ms current step. sEPSCs were recorded for 2 min at a holding potential of −80 mV. Following the acquisition of spontaneous synaptic data, tetrodotoxin (1 μM) was added to the Ringer's perfusion and, mEPSCs were recorded as above following a 5 min equilibration period. EPSCs/IPSCs were quantified using MiniAnalysis software (Synaptosoft), with an event detection threshold set at maximum RMS noise level (5 pA). The sEPSCs and sIPSCs were automatically detected and manually confirmed for mean frequency and amplitude. Averaged waveforms were created from all the EPSCs or IPSCs acquired for each neuron. From this, we calculated average EPSC and IPSC rise and decay time constants and area under the curve (pA/ms).

2.8 Neuron co-culture with FACS-isolated MIA E17.5 embryonic microglia live imaging

Mouse primary cortical neurons were isolated as previously reported (You et al., 2019.). Cortices of the embryonic mice at E16.5 were extracted in Hibernate-E medium (ThermoFisher) at 4°C. Tissues were chopped into 1-mm^3 pieces and digested with 0.25% trypsin-EDTA (ThermoFisher) for 15 min at 37°C and dissociated into single cells by gentle pipetting. Neurons were then plated onto PDL-coated 35 mm glass-bottom dishes with four micro-chambers (Ibidi) at a density of 1.0 x E4 cells per chamber in 20% fetal bovine serum (FBS) DMEM media (ThermoFisher) at 37C 5% CO2 incubator. 4 h later, the medium was replaced with complete neurobasal medium (Neurobasal medium, 2% B27, 2mM Glutamax). 50% media was refreshed every other day. At days in vitro (DIV) 3, neurons were transduced with CMV-rtTA (Addgene) and TetO-hNGN2-EGFP (Addgene) lentivirus. One day after the infection, 2 ug/ml doxycycline (Sigma-Aldrich) was added to the media to induce neuron differentiation and GFP expression.

At DIV14, embryonic murine CD11b+FCRLS+LY6C–microglia were isolated using FACS and cultured in complete neurobasal medium (ThermoFisher) supplemented with 20 ng/ml M-CSF1 (R&D System) (n = 10 pooled embryos from 1-2 litters from E17.5 embryonic brains derived of Saline or poly(I:C) injected dams). Microglia were plated at a density of 2.5 x 10E4 cells per chamber with DIV14 neurons. Complete neurobasal medium was refreshed ever two days and added 20 ng/ml murine M-CSF1 and 2 ug/ml doxycycline. At DIV19, live imaging was captured using confocal microscopy (LSM 880 with Airyscan, Zeiss) with a 63× oil objective at a resolution of

512 × 512. Images were deconvoluted using Autoquant for spine analysis. Spine density was quantified over a total dendritic length of about 50 μm using ImageJ software (n = 6–9 dendrites from 4–5 neurons per group). Filopodia subtypes were defined as protrusion length greater than 3 μm.

2.9 qRT-PCR validation

Total mRNA was extracted using the miRNeasy kit according to manufacturer's protocol (Qiagen). For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), 100ng of RNA was reverse transcribed and cDNA were obtained according to the manufacturer's protocols (SuperScript Vilo IV Kit; ThermoFisher). cDNA amplification and detection performed using commercially available FAM-labeled Taqman probes and TaqMan Universal PCR master mix (Applied Biosystems/Thermo Fisher Scientific). Real-time PCR reaction was performed using 7900HT PCR system (Applied Bio-systems). All qPCRs were performed in duplicate, and the data are either presented as relative expression compared to Gapdh as mean ± SEM or as fold change compared to the adequate control group.

2.10 Immunohistochemistry and immunocytochemistry

For biocytin-filled neuron immunohistochemistry, slices were fixed between filter papers in 4% PFA in 0.1 M PBS at pH 7.4 immediately after recording. Brain slices were fixed for 2 d at 4 °C, washed in PBS, permeabilized in 1% Triton X-100/PBS for 2 h at room temperature, then incubated with streptavidin-Alexa 488 (1:500; ThermoFisher) at

4 °C for 2 d. Slices were stored in PBS until processing by immunohistochemistry. Confocal imaging used confocal microscope Zeiss LSM710 and Zen 2010 software (Zeiss). For imaging the whole neuron, stacks were acquired using a 40 × /1.3 NA oil-immersion objective (Plan-Achroma, Zeiss) at a resolution of 0.2 × 0.2 × 0.5 μm per voxel. For magnified imaging of spine subtype density and interactions with microglia, image stacks were acquired using the oil 40x objective, at a resolution of 0.04 × 0.04 × 0.3 μm per voxel, from the basal dendritic branches (100 μm in length). All acquired stack of images was deconvolved using AutoQuant software (Media Cybernetics). Neuronal dendrites were manually traced, reconstructed, and analyzed using Neurolucida (MBF Bioscience).

For thin cryosectioning immunohistochemistry, mice were anesthetized with isoflurane and perfused transcardially with ice-cold PBS and 4% paraformaldehyde. Mouse brains were removed and fixed in 4% paraformaldehyde at 4C overnight. Fixed brains were dehydrated in 30% sucrose in PBS, snap-frozen in OCT, and stored in -80C until further processing. Brains were cut using cryosectioning, and 20 μm coronal sections containing prelimbic/infralimbic prefrontal regions were mounted on Superfrost Plus microscope slides (Fisher Scientific). The sections were subjected to antigen retrieval using sodium citrate at 90°C. For immunocytochemistry, cells were fixed at 4% paraformaldehyde for 15 min at room temperature. Samples were blocked in 5% normal donkey serum, then incubated with presynaptic marker-VGLUT2 (guinea pig, 1:500, Synaptic Systems, 135404), postsynaptic marker-PSD95 (goat, 1:500, Abcam, ab12093), and mononuclear phagocyte IBA1 (rabbit; Wako, 019-19741) diluted in 5% NDS, 0.2%

BSA, 0.1% Triton-X in PBS overnight. Sections were then rinsed with PBS and incubated in secondary antibodies (AlexaFluor546 donkey anti-goat secondary antibody; 1:1000, AlexaFluor647 donkey anti-guinea pig secondary antibody; 1:1000, and AlexaFluor488 donkey anti-rabbit secondary antibody; 1:1000) for 1 h at room temperature. Confocal microscopic imaging was performed on Zeiss LSM710, and z-stack images were acquired at z = 0.4 μm. All acquired images were deconvolved using Autoquant.

2.11 Spine density/subtype and microglia interaction analyses

For cell inclusion in neuron morphological analyses, cells must have intact soma and biotin-filled dendritic arbors. Spine subtypes were assigned based on published methods (Crimins et al. 2011). Spines were classified into four subtypes based on the width of the spine head and length of the spine neck: thin (head and neck <3 μm), stubby (no neck), mushroom (neck with head ≥0.6 μm), or filopodia (neck >3 μm in length). Microglia-spine and dendrite interactions were further quantified from the basal dendrites and classified based on the distance between neuron and microglia as follows: apposing (within 0.9 μm; three z-planes and microglia is surrounding less than half of the spine head diameter), and proximal (within 1.5 μm; five z-planes), and encapsulating (within 0.3 μm; one z-plane and microglia are surrounding at least ½ the spine head diameter) microglia-neuron interaction. The Pythagorean theorem was used to calculate microglia-neuron distance in 3D space, modeling interaction in the x, y, and z planes as equal to three sides of a right triangle. Microglial structures were manually traced by

experimenters blinded to the treatment groups using 8-bit confocal image stacks using NeuronStudio (CNIC, http://research.mssm.edu/cnic/tools-ns.html). Sholl analysis and branch order were quantified as previously described using NeuronStudio (Garay et al. 2013).

2.13 Data analysis

T-tests and ANOVAs were performed using Prism 8.0 or R, and correlation analysis were performed in JMP Pro 10. RNA-sequencing data were analyzed using R as described above. Pairwise correlation analyses were performed in JMP Pro 10 (SAS) to determine the correlation coefficient of Spearman's Rho. For all analyses, including correlation analyses, alpha values for confidence intervals were 0.05. Four-group comparisons were calculated using two-way ANOVAs, with main factors as poly(I:C) (MIA) and MG-REP (CSF1R inhibition treatment) or paired t-test within the group with Sidak method for adjusted p-values. For social behavior analysis, six group comparisons were performed using Three-way ANOVAs, with main factors poly(I:C) (MIA), MG-REP (treatment) and chamber time. For multiple comparisons, Tukey's posthoc multiple comparisons were implemented unless otherwise noted. Two-group comparisons between Saline and MIA were compared using unpaired t-test. Outliers were identified and removed using the ROUT test, with FDR=1%, in Prism. For microglia morphology, microglia were considered outliers if three or more values at individual branch orders were outliers by the ROUT test.

CHAPTER THREE

The beneficial effect of microglial repopulation in MIA

Disclaimer: Figures and portions of the text in chapter three were published as Ikezu, Yeh, Delpech, Woodbury *et al.* in Molecular Psychiatry (2020)

3.1 Introduction

Animal studies have shown that MIA can disrupt normal early neurodevelopment with a long-lasting impact on behavior and neuronal deficits associated with psychiatric disorders. Dysregulated cytokines were found in both neonates and adult MIA offspring in the frontal cortex and hippocampus, suggesting a long-lasting effect of prenatal immune challenge on neurodevelopment (Garay et al. 2013). Whether MIA can alter microglia remains controversial as findings have been variable across different labs and often contradictory (Smolders et al. 2018; Giovanoli et al. 2015). Some labs have reported no changes in microglia biology or cell density in MIA offspring (Smolders et al. 2015), whereas other labs have shown phenotypic changes in MIA microglia (Gumusoglu et al. 2017). Factors contributing to inconsistent findings include mouse strain, MIA inducing agent and timing, sex, age, and brain region analyzed (Kentner et al. 2019; Smolders et al. 2018). In rodent models, maternal immune activation can be achieved via immune-activating agents including live virus, lipopolysaccharide, a structural component of bacteria, and polyinosinic:polycytidylic acid (polyI:C), a synthetic analog of double-stranded RNA (Smith et al. 2007; Hsiao and Patterson 2011; Borrell et al. 2002).

However, the molecular mechanisms of how alterations to the microglial profile can cause behavioral abnormalities in MIA offspring remain elusive. We speculate that MIA can mediate social behavior deficits by causes changes in neuronal activity and microglia-neuron interactions. Therefore, we assessed the role of microglia in mediating neurodevelopmental changes due to maternal inflammation.

3.2 Results

MIA was induced by injection of 20 mg/kg poly(I:C) into pregnant C57BL6/J females on embryonic day 9.5 (E9.5) that corresponds to the initial wave of microglial migration from the yolk sac into the CNS (Ginhoux et al. 2010). At E9.5, MIA elicits a strong IL-6 response 3 h post-injection of poly(I:C) (**Figure 3.1a, b**), in agreement with previous literature suggesting IL-6 as the primary mediator of maternal inflammation (Smith et al. 2007). One group has shown that IL-17 may be critical in inducing MIA-deficits (Choi et al. 2016; Yim et al. 2017). However, IL-17 was not significantly upregulated by poly(I:C) 48 hours after injection in our model (**Figure 3.1c**), suggesting an MIA model independent of IL-17 when MIA was induced at an earlier stage or in a different environment. Early MIA also led to transient reduction of body weight at P21 weaning period (**Figure 3.2a**). But body weight was normalized later in adulthood (**Figure 3.2 b, c**). This suggests MIA may have a systemic but temporary impact during early development leading to weight loss around the adolescent period.

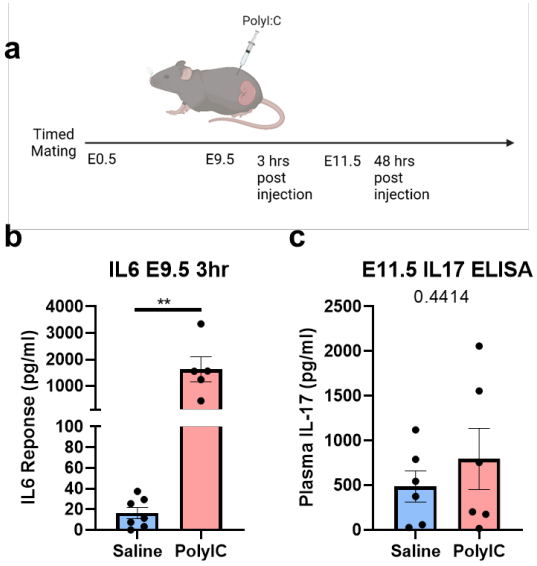


Figure 3.1. Maternal immune activation scheme and cytokine upregulation.

a) Experimental timeline of MIA induction in C57BL6 pregnant mice using Poly(I:C) and collection of blood plasma at 3 h and 48 h post injection. **b)** IL-6 ELISA of blood plasma of pregnant mice at 3 h post injection at E9.5. **c)** IL-17 ELISA of blood plasma at 48 hours post injection (n=5-6 pregnant females from two independent cohorts). Graphs indicate mean ± SEM. Data collection, analysis, and Figure by H Yeh.

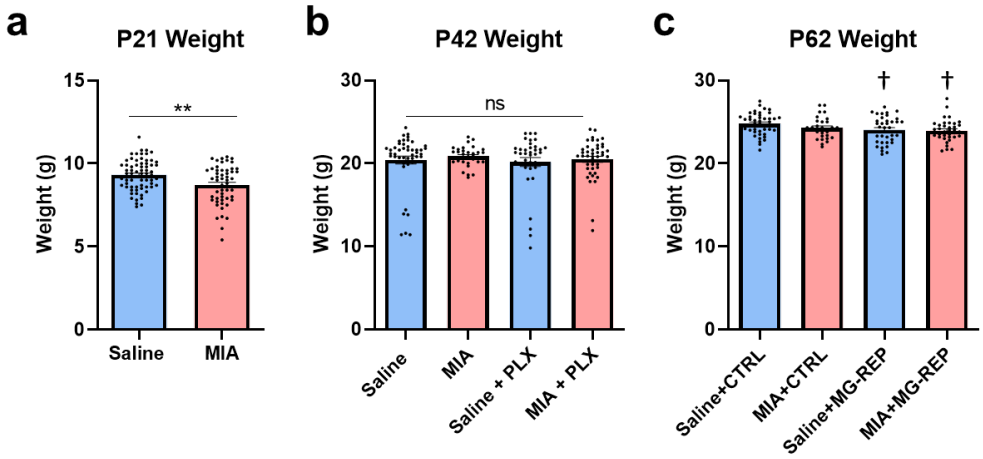


Figure 3.2. Weight gain over P21-62 of saline and MIA offspring.

a) MIA reduced weight at P21 (n of saline= 70, MIA= 55 males**). b)** no weight differences observed between all treatment groups at P42, when microglia are depleted in PLX-treated offspring (n=45/31/38/44**). c)** No MIA effect, but had significant PLX effect on P62 weight (n= 41/29/38/37). **p<0.005 as determined by student's unpaired t-test or Two-way ANOVA with Tukey's post hoc analysis, †p values for MG-REP treatment effect. ns: no significance. Graphs indicate mean ± SEM. Data collection by A. Van Enoo and H. Yeh. Analysis and figure by H. Yeh.

Here, we hypothesized that MIA-induced deficits in offspring could be alleviated by depletion and repopulation of microglia. Microglial depletion can be achieved through conditional knockout of microglial genes or colony-stimulating factor-1 receptor (CSF1R) pharmacological inhibitors. CSF1R is mainly expressed by microglia in the CNS, and CSF1R inhibition leads to microglial apoptosis and rapid removal from the brain (Elmore et al. 2014). Recent studies suggest that depleting microglia by CSF1R inhibition induces local repopulation from remaining microglia that are resistant to CSF1R inhibition (Huang et al. 2018; L. Zhan et al. 2019). Depletion and repopulation of microglia in aged mice alleviated age-related inflammation and reversal of cognitive and neuronal deficits and promoted neuroprotective microglia phenotype in response to brain injury (Elmore et al. 2018). In this study, microglia depletion occurred at P21-42, and CSF1R inhibitor (PLX) withdrawal led to microglia repopulation (MG-REP) in both Saline and MIA adult offspring (**Figure 3.3 a**). After three weeks of CTRL or PLX-containing diet, we found successful microglial depletion by PLX treatment in saline (99.8%) and MIA (99.9%) offspring at P42 and complete microglia replenishment at P60, 18 days after withdrawal of CSF1R inhibitor (**Figure 3.3 b, c**). MIA had no impact on microglial repopulation. Studies have shown PLX to have a minimal adverse effect on

health and behavior in mice (Elmore et al. 2014; 2018; Dagher et al. 2015). Bodyweight is an indicator of overall health. We found microglia depletion had no impact on body weight at P42 (**Figure 3.2b**), but MG-REP treated mice have reduced body weight compared to CTRL treatment mice later in P62 (**Figure 3.2c**). Therefore, these results demonstrate a transient reduction in body weight due to MIA and microglia repopulation following the withdrawal of CSF1R inhibition also reduces body weight regardless of MIA treatment. Further studies are required to determine if microglia repopulation treatment can affect appetite or other peripheral effects causing body weight reduction.

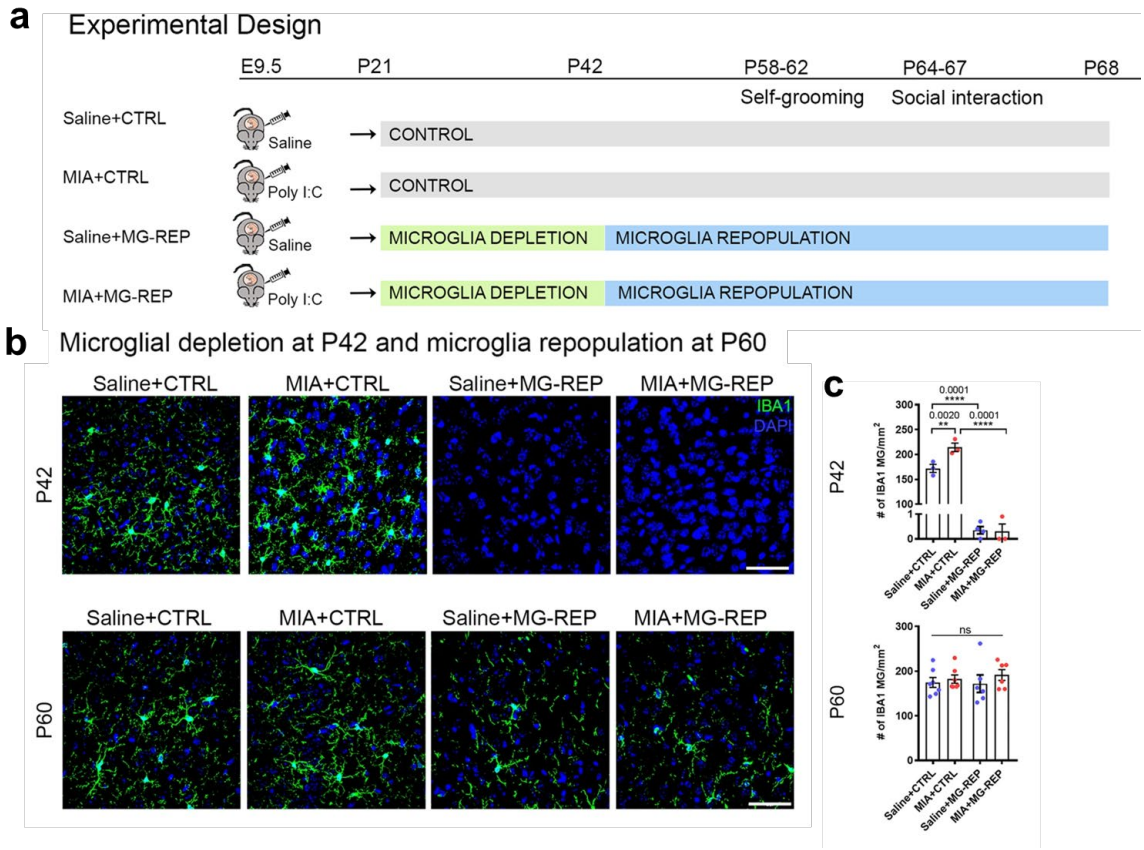


Figure 3.3. Microglial is removed by CSF1R inhibition, and the microglia density is normalized after microglia repopulation

a) MIA and CSF1R inhibition treatment experimental scheme. **b)** Representative images for microglial density with IBA1+ (green) and DAPI (blue) at P42 and P60 in adult male mPFC. Scale bar = 10 μm. **c)** Quantification of IBA1+ microglia in the mPFC showing microglial depletion by CSF1R inhibition at P42 (top) and repopulation microglia at P60 (bottom). n = (3/2, 3/2, 4/2, 3/2) for P42 male mice/litters; and n = (7/5, 7/5, 6/5, 6/3) P60 male mice/litters per group (Saline + CTRL, MIA + CTRL, Saline + PLX and MIA + PLX). Graphs indicate mean ± SEM. Data collection by JC Delpech and H Yeh. Analysis and Figure by H Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

Furthermore, we characterized the impact of MIA and microglia repopulation on social behavior in male offspring. MIA is known to induce social and communicative deficits, which are hallmarks of psychiatric disorders such as autism and schizophrenia (Malkova et al. 2012). Other MIA behavior deficits include impaired sensory gating such as prepulse inhibition, latent inhibition, and amphetamine-induced activity (Kobayashi et al. 2020.; Ballendine et al. 2015; Luan et al. 2018; Ozawa et al. 2006; Lins et al. 2019). Administration of the social three-chamber assay shows that Saline male mice spend more time in the chamber containing the novel mouse (S tube) (**Figure 3.4a-b**). In contrast, MIA + CTRL offspring show no preference for interacting with the novel mouse (**Figure 3.4a-b**). We found that microglia depletion and repopulation by a CSF1R inhibitor corrected social impairment in MIA offspring. We also examined self-grooming in male offspring for repetitive behavior testing at P58-62. MIA significantly increased the total self-grooming time, which was diminished by microglia repopulation treatment (**Figure 3.4c**). These results demonstrate microglial repopulation treatment may rescue MIA-induced social deficit and repetitive behavior.

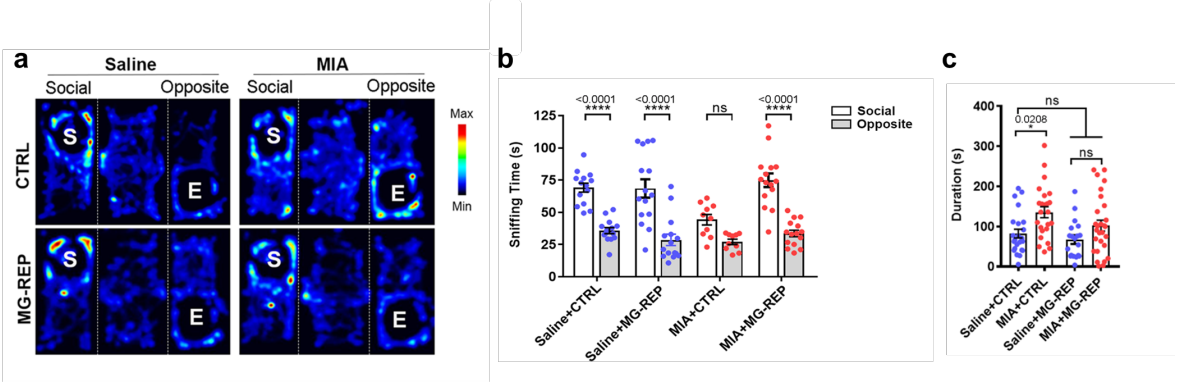


Figure 3.4. Microglia depletion and repopulation corrects social deficits and repetitive behavior in MIA offspring.

a) Representative heatmaps for Three Chamber Social Assay, "S": tube with social mouse, "E": empty tube. **b)** Total duration of time spent sniffing the "social" versus empty "opposite" cylinders in saline offspring (blue) and MIA offspring (red). n = (15/5, 15/9, 12/5, 15/4) male mice/litters for (Saline + CTRL, Saline + MG-REP, MIA + CTRL, MIA + MG-REP). Statistical significance determined by Three-way ANOVA (alpha = 0.05) and Sidak post-hoc. **c)** MIA male offspring display increased repetitive behavior. n = (23/9, 24/9, 19/11, 28/7) male mice/litters for (Saline + CTRL, MIA + CTRL, Saline + MG-REP, MIA + MG-REP). Determined by Two-way ANOVA and Tukey's post hoc. *p < 0.05; **p < 0.01; ****p < 0.0001, ns denotes no significance. Graphs indicate mean ± SEM. Behavior and analysis performed by JC Delpech. Figure by H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

To determine the effect of MIA on microglia, we quantified IBA1+ microglia density in the male offspring by immunohistochemistry of the medial prefrontal cortex (mPFC), the brain region mediating social behavior and previously implicated in aberrant neuronal circuitry and synchrony in MIA (Dickerson, Wolff, and Bilkey 2010). Consistent with previous literature, we found a transient increase of microglial density in the mPFC by MIA at P42 that was resolved by adulthood at P60 (**Figure 3.3 c**). However, increased microglial density has been reported in only a handful of studies, suggesting region-dependent and time-dependent manner (Smolders et al. 2018). Thus,

highlighting the critical window for microglia depletion and repopulation before maturity may be vital for successful intervention.

Another parameter used to assess the microglial phenotype is the expression of microglia markers IBA1 and P2RY12. IBA1 upregulation is associated with increased activation and P2RY12 is associated with homeostatic microglia. MIA has no impact on the expression of microglial markers IBA1 and P2RY12 in mPFC (**Figure 3.5 a-d**). In contrast, microglia repopulation caused a significant reduction in IBA1 and P2RY12 expression in mPFC, suggesting that PLX induces a more immature state with reduced IBA1 and P2RY12 expression at P60.

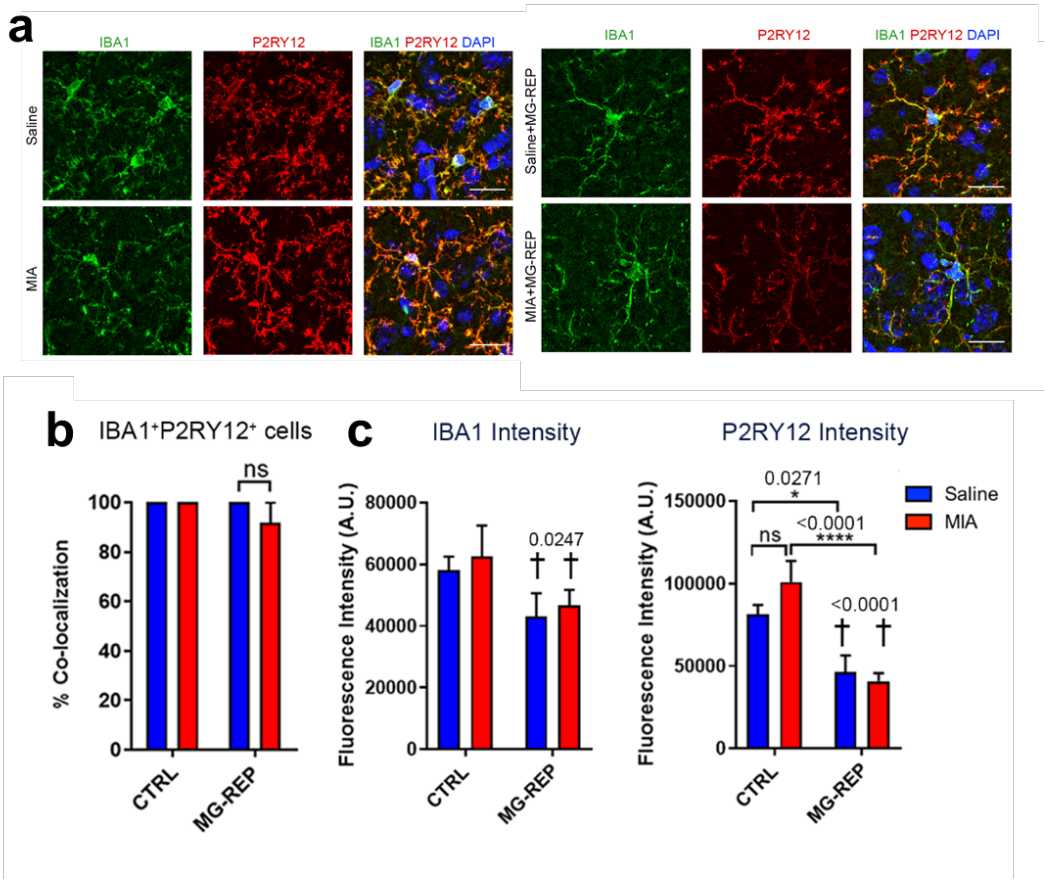


Figure 3.5. Microglial IBA1 and P2RY12 characterization.

a) Representative immunofluorescence of microglia as stained by IBA1 (green) and P2RY12 (red) and counterstained with DAPI for nuclear morphology in Saline or MIA offspring with or without microglial repopulation. Scale bar= 20 μm. **b)** Co-localization of IBA1+ and P2RY12+ cells with or without microglial repopulation in cortical region (n = 3 sections per group, female mice). **c)** Quantification of the fluorescent intensity of IBA1 (left) and P2RY12 (right) in microglia in the four treatment groups. n = (24, 18, 14, 24 cells) 2-3 male mice/ group for (P60 Saline+CTRL, MIA+CTRL, Saline+MG-REP and MIA+MG-REP). *$p < 0.05$ and ****$p < 0.0001$ as determined by Two-way ANOVA (alpha = 0.05) with Tukey's post-hoc. ns: no significance. †p values for treatment effect. Graphs indicate mean ± SEM. Data collection, analysis, and figure by H Yeh.

Upon pathological insults, microglia typically become activated with an amoeboid phenotype associated with increased secretion of proinflammatory cytokines (Hanisch and Kettenmann 2007). However, studies show that ramified microglia can be considered "active" and secrete comparable levels of proinflammatory and antiinflammatory cytokines similar to amoeboid microglia during development in healthy brain *in vivo* (Hinwood et al. 2013; Parakalan et al. 2012). MIA studies have shown contradictory results regarding microglia phenotype, with some studies showing increased ramification by MIA, some showing a reduced number of processes, and others showing no changes in microglia morphology in adult MIA offspring (Gumusoglu et al. 2017; Van den Eynde et al. 2014; Corradini et al. 2018; Chin W. Hui et al. 2018). We analyzed the microglial branching morphology in adult male offspring in layer V of the mPFC to assess the MIA effect on microglial morphology. 3D reconstruction of microglial processes with P2RY12 and IBA1 imaging analysis revealed microglia complex branches up to the 25th order (**Figure 3.6 a-b**). MIA significantly increased microglial ramification in MIA+CTRL compared to Saline+CTRL microglia, suggesting that MIA induces hyper-ramification of

microglial processes (**Figure 3.6 b,** red versus blue). Repopulated MIA microglia recovered normal branching morphology in MIA+MG-REP offspring (**Figure 3.6 b**, red versus magenta). This MIA-induced hyper-ramification suggest an alternative state of microglia and modified neuron-microglia interactions.

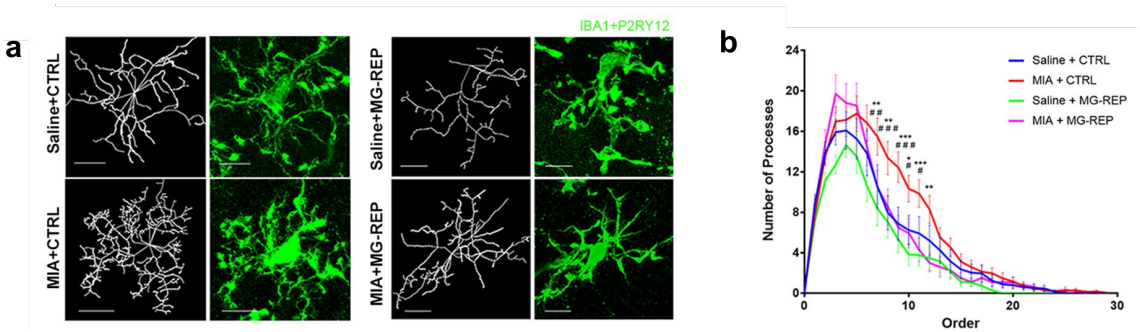


Figure 3.6. Morphological analysis of adult microglia reveals MIA-induced hyper-ramification.

a) Representative 3D reconstruction traces and P2RY12+IBA1+ (green) microglia confocal images. Scale bar = 10 µm. **b)** Microglial branch quantification at P60. n = (25/6/4, 36/5/4, 24/6/4, 20/6/5) (cells/male mice/litters) for Saline + CTRL, MIA + CTRL, Saline + MG-REP, MIA + MG-REP. One, two, and three * or # symbols denote $p < 0.05$, 0.01, 0.001, between MIA + CTRL vs. Saline + CTRL or MIA + MG-REP by Two-way ANOVA and Tukey's post-hoc, respectively. Data collection by M. Woodbury and H Yeh. Analysis by A Van Enoo, M. Woodbury, M. Bostros. Figure by H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

To characterize microglial transcriptomic changes underlying MIA phenotype and microglia repopulation effect, we performed RNA-sequencing on CD11b+ microglia from Saline and MIA offspring at E17, P7, P20, and P60 (**Figure 3.7**). To verify the purity of RNA-seq across different ages and treatments, we determined the relative expression of cell type-specific markers using published datasets of murine microglia, astrocytes, neurons, oligodendrocytes, and endothelial cells (Zhang et al. 2014). We

found an average of 98% purity of microglia markers, consistent with results from Zhang et al. database (97.8%) and the FACS-purified microglia RNA-seq database (98%) (Solga et al. 2015). We also evaluated sex as a potential confounding factor but found no significant results of sex-modified treatment effects.

We identified five clusters of genes associated with distinct developmental stages and MIA phenotype using K-means clustering of 14,225 genes of our microglial RNA-seq results **(Figure 3.7a** and **Table 3.1)**. The five clusters with distinct transcriptional signatures consist of immature microglia (IM) module genes enriched at E17 and P7, MIA immature microglia (MIA-IM) module genes enriched in MIA E17 and P7 offspring, juvenile microglia (JM) genes enriched in P20 Saline and MIA offspring, adult microglia (AM) module genes enriched in P60 Saline + CTRL offspring, MIA + CTRL and MIA + MG-REP offspring, and repopulated adult microglia (REP-AM) module genes enriched in P60 Saline + MG-REP offspring.

Microglia repopulation had a significant effect on adult saline offspring as indicated by the enrichment of genes identified in the REP-AM module, suggesting reprogrammed gene expression profiles of repopulated microglia (**Figure 3.7a**). Analysis of microglial gene signature revealed that Saline + MG-REP microglia express enrichment of genes associated with DAM/MGnD phenotype, including increased expression of *Cstb* and reduced expression of *Tmem119* at P60 (**Figure 3.7c**). In contrast, MIA + MG-REP showed a homeostatic phenotype like Saline+CTRL microglia (**Figure 3.7b-c**). These results reveal changes in the microglial composition during repopulation under MIA influence. The newly replenished microglia in Saline + MG-REP offspring

show DAM/MGnD phenotype, characterized by enrichment of genes associated with phagocytic function, chemotaxis, and proliferation, and is reminiscent of immature microglial phenotype (Krasemann et al. 2017; Li et al. 2019; Keren-Shaul et al. 2017). The discrepancy of repopulation phenotype between Saline and MIA offspring suggests an accelerated maturation of microglia, which was also demonstrated by a previous study, or more resistance against microglia repopulation-induced changes in MIA + MG-REP offspring (Matcovitch-Natan et al. 2016).

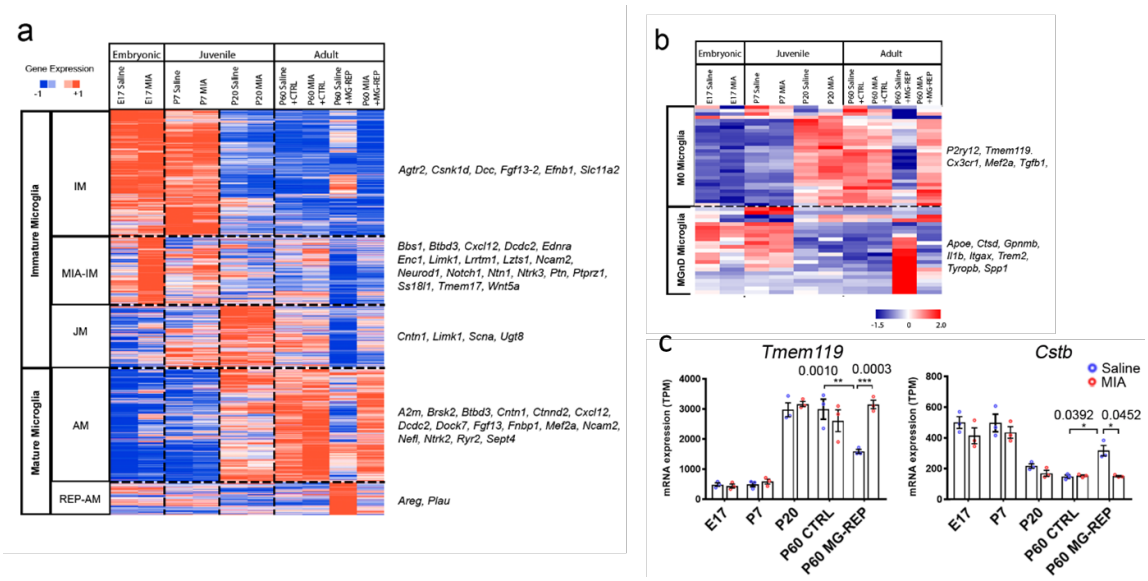


Figure 3.7. Microglial transcriptome reveals MIA and repopulation modules

a) Heatmap of differentially expressed genes in acutely isolated microglia categorized into five modules at different time points: immature microglia (IM), MIA immature microglia (MIA-IM), juvenile microglia (JM), adult microglia (AM), repopulated adult microglia (REP-AM). n = 3 pooled microglial samples per group. For E17, one litter of 4-5 embryos per sample. For P7, three pooled brains per sample. For P20 and P60, one brain per sample. **b)** Heatmap of differentially expressed genes in acutely isolated microglia in M0 (homeostatic microglia) and DAM/MGnD-specific genes based on z-scores. **c)** Normalized expression of microglial *P2ry12* and *Cstb* across different treatments and time points. * and ** denote $p < 0.05$ and 0.01 as determined by two-way ANOVA and Tukey's posthoc. Data collection by M. Woodbury. Analysis by A. Desani and W. Johnson. Figure by H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

	M0 genes	**MGnD/DAM genes**	**Immature genes**
IM	*Spi1*	*Bhlhe40, Cstb, Spp1, Gnas, Gpnmb*	*Ctsz, Hmox1, Igfbp4, Casp4, Nlrp3*
MIA-IM	*Egr1*	*Arg1*	*Wnt5a, Enc1, Cirbp, Ptn*
JM	*P2ry12, Csf1r, Mef2a, Cx3cr1, Mertk*	*Csf1*	*Ccl4, Fkbp5, Atf3, Ugt8a*
AM	*Mafb, Tgfb1, Adgrg1, Tmem119*	*Ctsd, Trem2*	*Atp1a2, C1qa, C1qb, C1qc, Dusp1, Vamp2, Ptgds, Scd2, Pea15a, Fcgr3, Fos*
REP-AM	*Il10rb*	*ApoE, Lyz2, Cst7, Itgax, Cybb, Grn, Il1b, mir155, Tyrobp, Ccl2*	*Ntn, B2m, Cd14, Cxcl2, Ifi30, Lgals3bp, Psme1, Tlr2*

Table 3.1. Enrichment of genes based on modules and microglia phenotype

Recent studies reported the *in situ* expansion of microglia from remaining microglia after removal of CSF1R inhibitor (Huang et al. 2018; L. Zhan et al. 2019).

Zhan et al. identified a subset of MAC2+ microglial population resistant to CSF1R inhibition and acts as the source for repopulation (Zhan et al. 2020). To further evaluate the MIA effect on microglial repopulation source, we performed a fate-mapping analysis of microglia cells using thymidine analog bromodeoxyuridine (BrdU) and 5-Ethynyl-2′-deoxyuridine (EdU), as described in Chapter 2 Methods (**Figure 3.8a**). Microglia continue to expand until three weeks of age, enabling BrdU labeling of postnatal resident microglia proliferative between P1 and P20 (Nikodemova et al. 2015; I. Kim et al. 2015). Immunohistochemistry was performed to quantify IBA1 colocalization with cell proliferation markers BrdU and EdU to assess if newly repopulated EdU+ IBA1+ microglia arise from previously mitotic BrdU+ IBA1+ microglia or previously non-proliferative cells BrdU−IBA1+microglia (**Figure 3.8a-b**). BrdU labeling efficiency for Saline + CTRL and MIA + CTRL were 41.65% and 35.13%, respectively, of total IBA1+ microglia (**Table 3.2**). MIA significantly suppressed the relative proportion of original repopulated BrdU+EDU+ microglia, suggesting MIA reduced microglial self-renewal in adulthood (**Figure 3.8c**, middle, **Table 3.3**). Previously non-proliferative BrdU− IBA1+ microglia (blue) were the primary source of repopulation in MIA + MG-REP offspring (67%). In contrast, a higher proportion of microglia repopulated from previously proliferative BrdU+ microglia (green) in Saline + MG-REP offspring (**Figure 3.8d**). Saline+MG-REP microglia show enrichment of genes associated with cell proliferation and early developmental genes resembling immature or DAM/MGnD microglia profile (**Figure 3.7b**), which suggests that previously proliferative BrdU+ microglia maintain mitotic activity after repopulation in Saline offspring. In contrast, MIA+MG-REP

microglia were mostly repopulated from previously non-mitotic BrdU− microglia, which could revert to a non-proliferative status resembling homeostatic (M0) microglia (**Figure 3.8c-d**).

Normalized by IBA1+	BrdU+EDU- IBA1+	BrdU+EdU+ IBA1+	BrdU-EdU+ IBA1+	Total Labeled Cells
Saline+CTRL	36.16%	4.38%	1.11%	41.65%
MIA+CTRL	34.46%	0.67%	0.00%	35.13%
Saline+MG Rep	10.55%	18.40%	15.45%	44.40%
MIA+MG Rep	4.22%	7.26%	27.47%	38.94%

Table 3.2. Labeling efficiency of IBA1 microglial cells

Relative Percentage of Labeled MG	BrdU+EDU- /IBA1	BrdU+EdU+ /IBA1	BrdU-EdU+ /IBA1
Saline+CTRL	86.83%	10.50%	2.67%
MIA+CTRL	98.10%	1.90%	0.00%
Saline+MG Rep	23.77%	41.44%	34.79%
MIA+MG Rep	10.83%	18.64%	70.53%

Table 3.3. Relative proportion of BrdU, BrdU/EdU+ and EdU+ cells per treatment

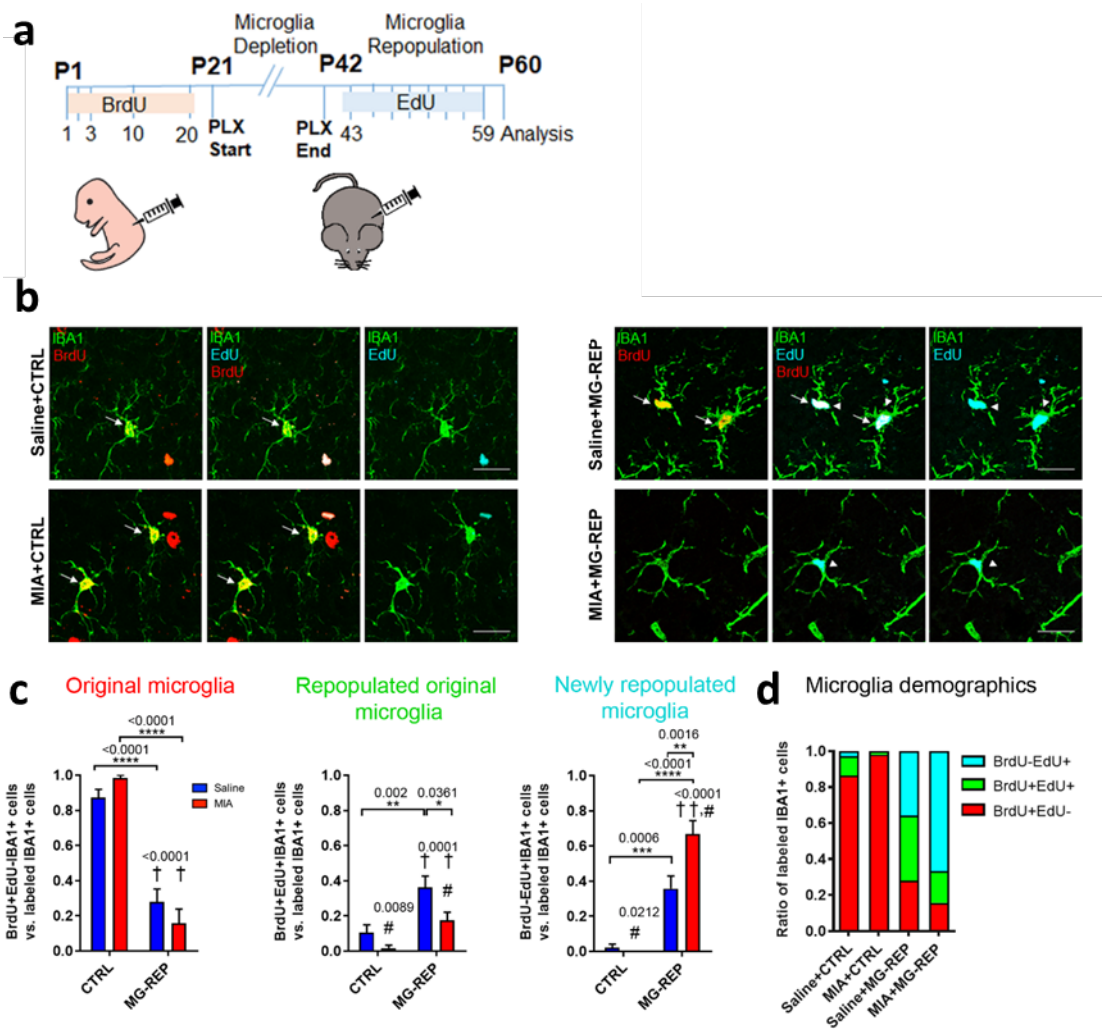

a
Microglia Depletion
Microglia Repopulation
P1
P21
P42
P60
BrdU
EdU
1 3 10 20 PLX Start
PLX End 43
59 Analysis
b
Saline+CTRL
MIA+CTRL
Saline+MG-REP
MIA+MG-REP
IBA1
BrdU
EdU
c
Original microglia
Repopulated original microglia
Newly repopulated microglia
BrdU+EdU-IBA1+ cells vs. labeled IBA1+ cells
Saline
MIA
CTRL
MG-REP
d
Microglia demographics
Ratio of labeled IBA1+ cells
BrdU-EdU+
BrdU+EdU+
BrdU+EdU-
Saline+CTRL
MIA+CTRL
Saline+MG-REP
MIA+MG-REP

Figure 3.8. Fate mapping study analysis shows distinct population of repopulated microglia

a) Schematic illustration of thymidine analog BrdU and EdU injections to P1 to P59 Saline and MIA offspring. BrdU and EdU were injected in a total of 5 (P1, 2, 3, 10 and 20) and 8 times (every other day from P43-59), respectively. **b)** Representative images of BrdU (red), EdU (cyan), and IBA1 (green) microglia for Saline+CTRL (**b**, top left), MIA+CTRL (**b**, bottom left), Saline+MG-REP (**b**, top right) and MIA+MG-REP (**b**, bottom right). Arrow indicates BrdU+IBA1+ original microglia. Arrowhead points to EdU+IBA1+ repopulated microglia. Scale Bar = 20 um. **c)** Fate mapping analysis of BrdU+EdU−IBA1+ original microglia in cortex (c, left). Reduced repopulation of microglia (BrdU+EDU+IBA1+) in MIA+MG-REP offspring (c, middle, MIA effect $p = 0.0089$), and significantly increased newly repopulated microglia (BrdU-EDU+) in MIA+MG-REP offspring (**c**, right). #$p < 0.05$ for MIA effect, †$p < 0.05$ for Treatment effect. **d)** Relative distribution of all microglial in Saline and MIA cortex at P60. n = (19, 12, 18, 20) female mice for Saline+CTRL, MIA+CTRL, Saline+MG-REP, MIA+MG-REP. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$; determined by Two-way ANOVA (alpha = 0.05) and Tukey's post-hoc. Graphs indicate mean ± SEM. Data collection by H. Yeh and A. Van Enoo. Analysis and figure by H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

3.3 Discussion

In our MIA model, we found significant IL-6 induction by poly(I:C) injection at E9.5. The lack of IL-17 upregulation may be due to the timing of MIA induction and mouse strain lacking commensal segmented filamentous bacteria (Kim et al. 2017). This study reveals that MIA can be induced independently of IL-17 induction at earlier stages and elicit consistent impairment in sociability and transient microglial alterations. Microglia depletion can be achieved by CSF1R inhibition in both Saline and MIA offspring. Microglia phenotypes change over time due to specific functions at certain periods (Grabert et al. 2016; Thion et al. 2019; Matcovitch-Natan et al. 2016). We found a transient increase in microglia density at P42 due to MIA. Our findings correspond to

previous studies showing increased microglial density in the hippocampus of MIA offspring between P2 and P30 but no longer present in later adulthood (Li et al. 2014; Manitz et al. 2013). Consequently, MIA-induced deficits in microglia can be age-dependent. Our results demonstrate that MIA can induce microglial changes consisting of transient increased microglial density, hyper-ramification, and reduced self-renewal and transcriptome changes corresponding to an accelerated maturation phase of microglia previously reported (Gosselin et al. 2016). Furthermore, microglia surface markers from P30 mice showed MIA-induced changes that were lost at P100 (Eßlinger et al. 2016). The time-specific MIA-induced microglia changes can be due to the age-specific function of microglia, such as synapse pruning and remodeling of brain circuitry at the peak of synaptogenesis during development (Mosser et al. 2017). Microglia can alter their functions to adapt to different requirements in the developing brain.

This suggests that maternal inflammation can have a long-lasting impact on the microglia phenotype corresponding to behavioral deficits. Previous studies have shown that reducing inflammation by exercise or minocycline treatment can also alleviate MIA-induced behavior deficits (Mattei et al. 2017; Andoh et al. 2019). This study highlights that microglia could play a critical role in mediating MIA-induced deficits. Microglia depletion and repopulation by CSF1R inhibition led to the correction of MIA-induced deficiencies. In addition, repopulation of microglia led to normalized morphology and transcriptome in MIA offspring. However, microglia depletion and repopulation seemed to induce an immature phenotype that is the most prominent in SAL+MG-REP mice, highlighting the MIA effect on microglial self-renewal. In addition, due to oral

administration, CSF1R inhibition may reduce specific subtypes of peripheral immune cells, including resident and interstitial macrophages of peritoneum, lung, and liver expressing CSF1R (Lei et al. 2020). This may be further evaluated in the MIA model to determine if modulating peripheral immune cells can contribute to the correction of MIA phenotype via other pathways such as the gut-brain axis.

CHAPTER FOUR

Microglia transcriptome altered by MIA and microglia repopulation

Disclaimer: Figures and portions of the text in Chapter Four were published as Ikezu, Yeh, Delpech, Woodbury *et al.* in Molecular Psychiatry (2020)

4.1 Introduction

Maternal inflammation can change the microglial transcriptome, including molecular pathways involved in synaptogenesis and neuronal network formation critical to developmental functions of microglia (Pont-Lezica et al. 2014; Squarzoni et al. 2014; Matcovitch-Natan et al. 2016; Mattei et al. 2017). Matcovitch-Natan et al. demonstrated that MIA shifts the developmental trajectory of microglia to be more mature and thereby altering microglial functions in the offspring of MIA (2016). Accelerated development of microglia was also identified in ASD brain (Hanamsagar et al. 2017). However, the effect of this premature microglia maturation on neurodevelopment remains unclear.

Several other studies show MIA has a wide range of impact on neurons including increased phagocytosis of neuronal progenitor cells, decreased outgrowth of dopaminergic axons, and defasciculation of dorsal callosal axons (Cunningham, Martínez-Cerdeño, and Noctor 2013; Squarzoni et al. 2014; Pont-Lezica et al. 2014). MIA also increased microglial IL-6 expression and microglial motility, suggesting subtle changes in microglial immune surveillance function (Smolders et al. 2018; Ozaki et al. 2020). Microglia motility in the developing brain is proposed to be mediated by trophic signals (Mosser et al. 2017; Ozaki et al. 2020). These results indicate that early maternal

inflammation can dysregulate microglial development and impair critical microglial functions in supporting neuron development and synaptic plasticity.

4.2 Results

We found discrete and overlapping genes significantly altered by MIA at each age (**Figure 4.1**: 666 (E17), 225 (P7), 312 (P20), 161 (P60) unique genes MIA versus Saline with $p < 0.05$). These MIA-induced gene expression changes provide evidence for lifelong disruptions of microglial transcriptome by MIA. Further examination shows a minimal overlap between the distinct stages of development of MIA-induced gene expression changes, suggesting that MIA alteration of microglia transcriptome is age-dependent (**Figure 4.1**). The 14 common genes between E17 and P7 elements include *Scara5*, a ferritin receptor mediating iron delivery. Abnormal expression of *Scara5* can indicate MIA leads to iron accumulation in E17 and P7 microglia and suggesting an activated state due to MIA since iron accumulation has been reported in activated microglia (Kenkhuis et al. 2021; McIntosh et al. 2019). *Irf9* mediates type I interferon signaling, representing sustained poly(I:C)-induced antiviral response in microglia during these periods. The 19 common genes between E17 and P20 include zinc finger transcriptional factors (*Zfp398*, *Zfp467*), iron transport (*Slc11a2*), multiple drugs and metal transport (*Slc47a1*), synaptic molecules (*Sv2c*, *Shisa6*), and exocytosis (*Clec3b*). The shared differential gene expression suggests MIA-induced dysregulation of active transport of iron and metals, exocytosis, and modulation of synaptic activities in early and postnatal microglia. The 10 common genes between E17 and P60 include immune-

related chemokine and TGFB signaling (*Ccl2*, *Tgif1*) and inhibition of sodium-coupled chloride cotransporter (*Wnk2*). The only common gene among E17, P7, and P60 *Ankrd53* is related to mitosis. The 10 common genes between P20 and P60 include transcription factors (*Egr2*, *Prox2*, *Prrx2*), mTORC2 pathway (*Prr5*), mitosis (*Ptp4a1*), and novel transmembrane proteins (*Tspan4*, *Tmem132a*). However, further studies are necessary to understand how MIA-induced and age-dependent changes in these molecules can alter microglia function during development and adulthood.

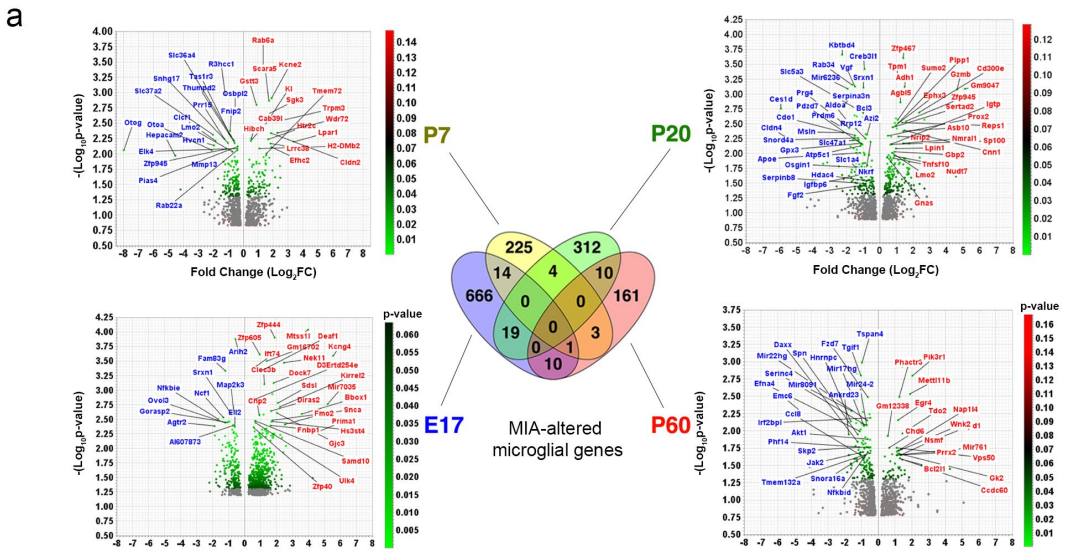


Figure 4.1 Differentially expressed genes of MIA versus Saline microglia across development

a) Venn diagram and volcano plots of significantly differentially expressed genes (DEG) in MIA versus Saline microglia at each age. Venn diagram shows number of genes with $p < 0.05$ between MIA and Saline microglia by exact t-test. Volcano plots: significantly differentially expressed genes between MIA+CTRL and Saline+CTRL. Red text: genes upregulated; blue text: genes downregulated by MIA. X-axis: log2 fold change in MIA+CTRL versus Saline+CTRL microglia, Y-axis: inverse log (p-value). Grey dots denote genes with $p \geq 0.05$. Data collection and analysis by M. Woodbury and A. Desani. Figure by M. Woodbury and H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

To further understand the molecular changes induced by MIA and microglial repopulation, we utilized gene ontology (GO) enrichment analysis to identify biological process associated with MIA-altered and microglia repopulation normalized genes. The most significant enriched GO pathways of MIA-induced and MG-REP-corrected genes were associated with synaptic vesicles and neurotransmission, including excitatory and inhibitory synaptic signaling pathways (**Figure 4.2a**, left). In contrast, genes suppressed by MIA and corrected by microglial repopulation were associated with antigen presentation, complement activation, and innate immune response (**Figure 4.2a**, right). MIA microglia express enrichment of the genes related to the formation of cellular protrusions and neuritogenesis, while repopulated microglia recover immune-related functions. These results reveal an alternate role of microglia in supporting neuritogenesis under the influence of maternal inflammation, which can be alleviated by microglial repopulation.

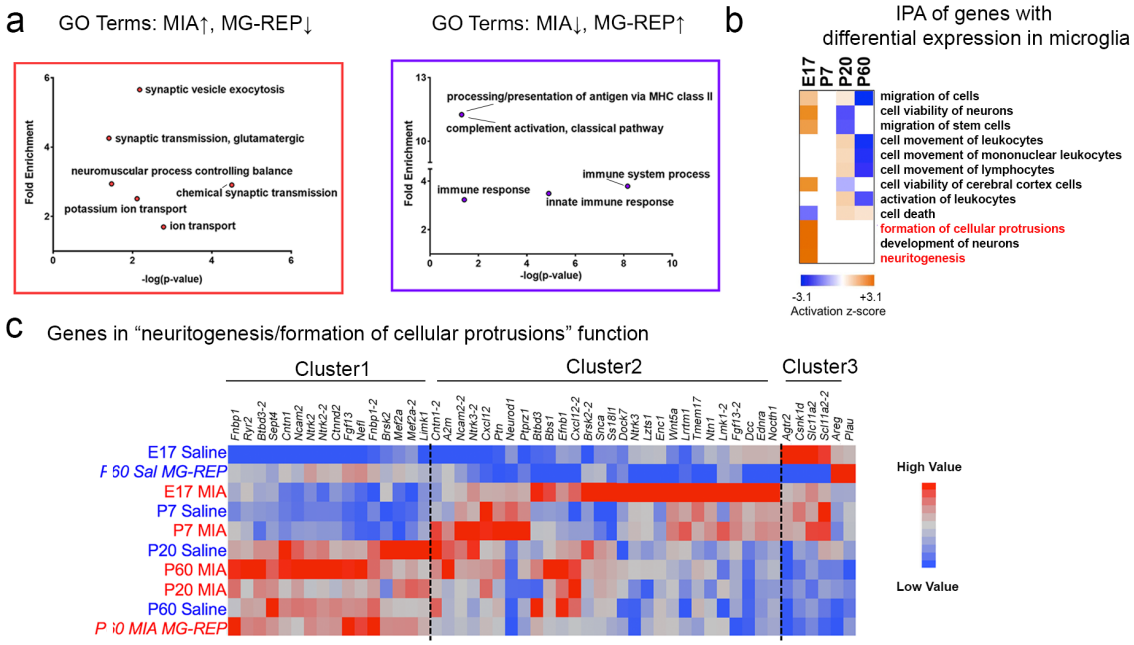


Figure 4.2. Microglia repopulation reduced expression of cellular protrusion/neuritogenic molecules in MIA microglia.

a) Gene ontology (GO) biological processes pathway. Left (red): Top significantly enriched GO terms of biological process increased by MIA and decreased by microglia repopulation. Right (purple): Top significantly enriched GO terms of biological process decreased by MIA and normalized by microglia repopulation. **b)** IPA results of microglial differential gene expression comparing MIA versus Saline. Red denotes significant biological pathways activated by MIA in E17 microglia. **c)** Heatmap showing genes enriched in "neuritogenesis/formation of cellular protrusions" signaling pathways. Hierarchal clustering of three gene sets based on relative expression values (red: highest versus blue: lowest expression). Data collection and analysis by M. Woodbury. Figure by M. Woodbury and H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

To further determine how MIA-induced microglial transcriptional changes at each time point, we analyzed biological processes significantly affected genes by MIA at each age using Ingenuity Pathway Analysis (IPA) (**Figure 4.2b**). Pathways involving "formation of cellular protrusions" and "neuritogenesis" were the two most significantly

enriched biological processes in E17 MIA microglia (**Figure 4.2b**). During development or injury response, microglia produce trophic factors including insulin-like growth factor-1 (IGF-1), thrombospondin, or brain-derived neurotrophic factor (BDNF), leading to maturation of neuronal circuit or promoting brain repair (Choi et al. 2008; Madinier et al. 2009). This MIA-induced enrichment of neuritogenesis genes, which describes a cluster of genes that promote neurite growth and synaptic plasticity, reflects a dysregulated function of microglia for supporting neurodevelopment.

We identified three clusters using hierarchical clustering of the genes to better understand MIA-altered neuritogenic pathways in microglia (**Figure 4.2c**). Cluster 1 contained genes increased in adult MIA microglia but reduced by microglia repopulation, including cell adhesion molecules *Ctnnd2* (catenin delta 2) (Turner et al. 2015), *Ncam2* (neuronal cell adhesion molecule 2 and enhancer of filopodia formation) (Sheng, Leshchyns'ka, and Sytnyk 2015), and *Ntrk2* (neurotrophic receptor tyrosine kinase 2 and TrkB receptor for BDNF). Cluster 2 genes consisted of genes upregulated by MIA in immature microglia, including *Ncam2*, *Ntn1* (netrin1 and an axon guidance molecule), *Ptn* (pleiotrophin and a secretory growth factor), and *Wnt5a* (wingless-type MMTV integration site family member 5A and synaptogenic molecule) (Varela-Nallar et al. 2010). Cluster 3 contained genes downregulated by MIA in immature microglia, including *Plau*. These clusters reveal sets of specific neuritogenic genes upregulated by MIA at distinct developmental periods, and a subset of neurogenic genes can be normalized by MG-REP treatment.

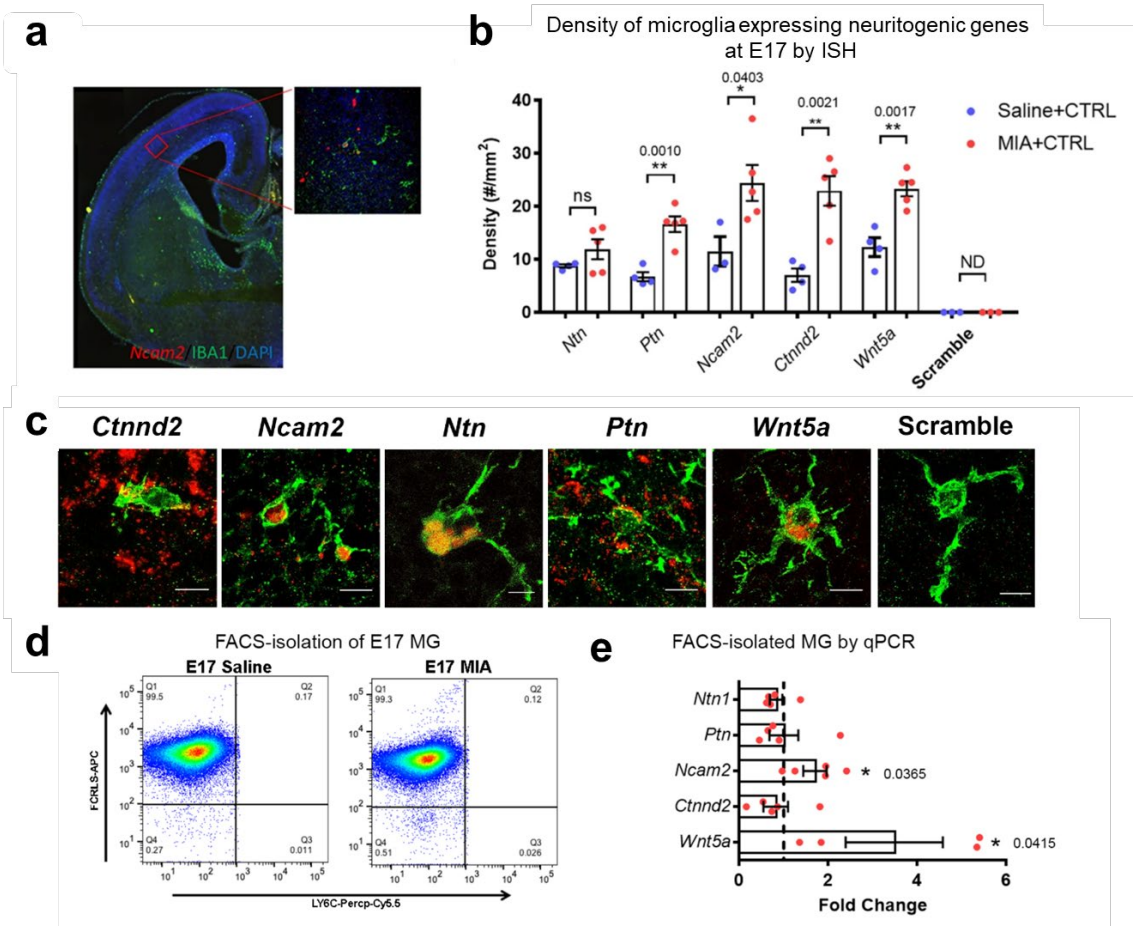

a
Ncam2 IBA1 DAPI
b
Density of microglia expressing neuritogenic genes at E17 by ISH
Density (#/mm2)
Saline+CTRL
MIA+CTRL
ns
0.0010
0.0403
0.0021
0.0017
ND
Ntn
Ptn
Ncam2
Ctnnd2
Wnt5a
Scramble
c
Ctnnd2
Ncam2
Ntn
Ptn
Wnt5a
Scramble
d
FACS-isolation of E17 MG
E17 Saline
E17 MIA
FCRLS-APC
LY6C-Percp-Cy5.5
e
FACS-isolated MG by qPCR
Ntn1
Ptn
Ncam2
Ctnnd2
Wnt5a
0.0365
0.0415
Fold Change

Figure 4.3 Validation of MIA-induced upregulation of neurogenic genes in embryonic microglia

a) Representative images of the confocal microscopic images of the *in situ* hybridization (ISH) and immunofluorescence of E17.5 IBA1+ (green) male microglia. **b)** Quantification of *in situ* hybridization was performed for detecting mRNA of *Ptn*, *Ntn*, *Ctnnd2*, *Ncam2*, *Wnt5a*, and scramble control (red) in IBA1 (green) microglia. **c**) Magnified representative images of specific probes (red) *Ptn*, *Ntn*, *Ctnnd2*, *Ncam2*, *Wnt5a*, and scramble control, and IBA1+ (green) microglia. Scale bar = 5 μm. **d**) Assessment of neuritogenic molecules in FACS-purified microglia from E17 Saline and MIA embryonic brain. FCRLS+ LY6C– microglial population (Q1) were 99.5% in Saline+CTRL group and 99.3% in MIA+CTRL group of gated CD11b+ cells. **e)** The changes in neuritogenic gene expression (*Ptn*, *Ntn*, *Ctnnd2*, *Ncam2* and *Wnt5a*) in E17 microglia were determined by qPCR and shown as fold change in MIA+CTRL over Saline+CTRL group. N = 5 litters per group (total 25 pups for Saline+CTRL and 27 pups for MIA+CTRL groups). *$p < 0.05$ as determined by unpaired Student t-test. Graphs indicate mean ± SEM. Data collection by JC Delpech, H. Yeh, and Y. You. Analysis by JC Delpech, M. Bostros and S. Hersch. Figure by JC Delpech and H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

To validate microglial expression of cellular protrusion factors, we combined *in situ* hybridization with immunofluorescence detected specific gene expression in IBA1+ embryonic microglia (**Figure 4.3a-c**). We detected several cellular protrusion molecules in IBA1+ microglia cells in cortical plate region of E17 brain using DIG-labeled RNA probes, while negative control using the scramble RNA probe lacked any signal (**Figure 4.3c**). Furthermore, the number of IBA1+ embryonic microglia expressing *Ctnnd2*, *Ncam2*, *Ptn*, and *Wnt5a* in the cortical plate region were significantly higher in MIA compared to Saline offspring, validating several mRNA expression changes induced by MIA identified in our RNA-seq dataset (**Figure 4.3b**).

We also confirmed MIA-induced upregulation of select neuritogenic genes in FACS-purified E17.5 microglia to eliminate the possibility of cell contamination during

microglia isolation by MACS CD11B+. FCRLS+ LY6C– microglial population (Q1) were 99.5% in Saline+CTRL group and 99.3% in MIA+CTRL group of gated CD11b+ cells, suggesting no significant changes in FCRLS+ microglia population due to MIA (**Figure 4.3d**). Here, we show significant increases in the mRNA expression levels of *Ncam2* and *Wnt5a* in MIA microglia (**Figure 4.3e**). The changes in neuritogenic gene expression (Ptn, Ntn, Ctnnd2, Ncam2, and Wnt5a) in E17 microglia were determined by qPCR and shown as fold change of MIA+CTRL over the Saline+CTRL group.

In addition to mRNA level validation, we confirmed MIA-induced upregulation and MG-REP normalization of select neuritogenic molecules including CTNND2, NCAM2, and NTRK2 using ELISA of P60 microglia isolated from Saline and MIA female offspring (**Figure 4.4a-c**). Although MIA did not increase protein expression level in NTN and PTN, CSF1R inhibition reduced PTN expression in Saline + MG-REP and MIA+MG-REP offspring compared to CTRL offspring (**Figure 4.4d-e**). MIA significantly increased the microglial WNT5A protein expression. However, microglia repopulation did not normalize MIA-induced upregulation of WNT5a, suggesting CSF1R inhibition did not affect microglial WNT5a expression in adult offspring (**Figure 4.4f**). These results indicate that MIA-induced upregulation of neurogenic genes CTNND2, NCAM2, and PTN can be detected in embryonic and adult microglia, suggesting a long-lasting effect of MIA on microglial profile.

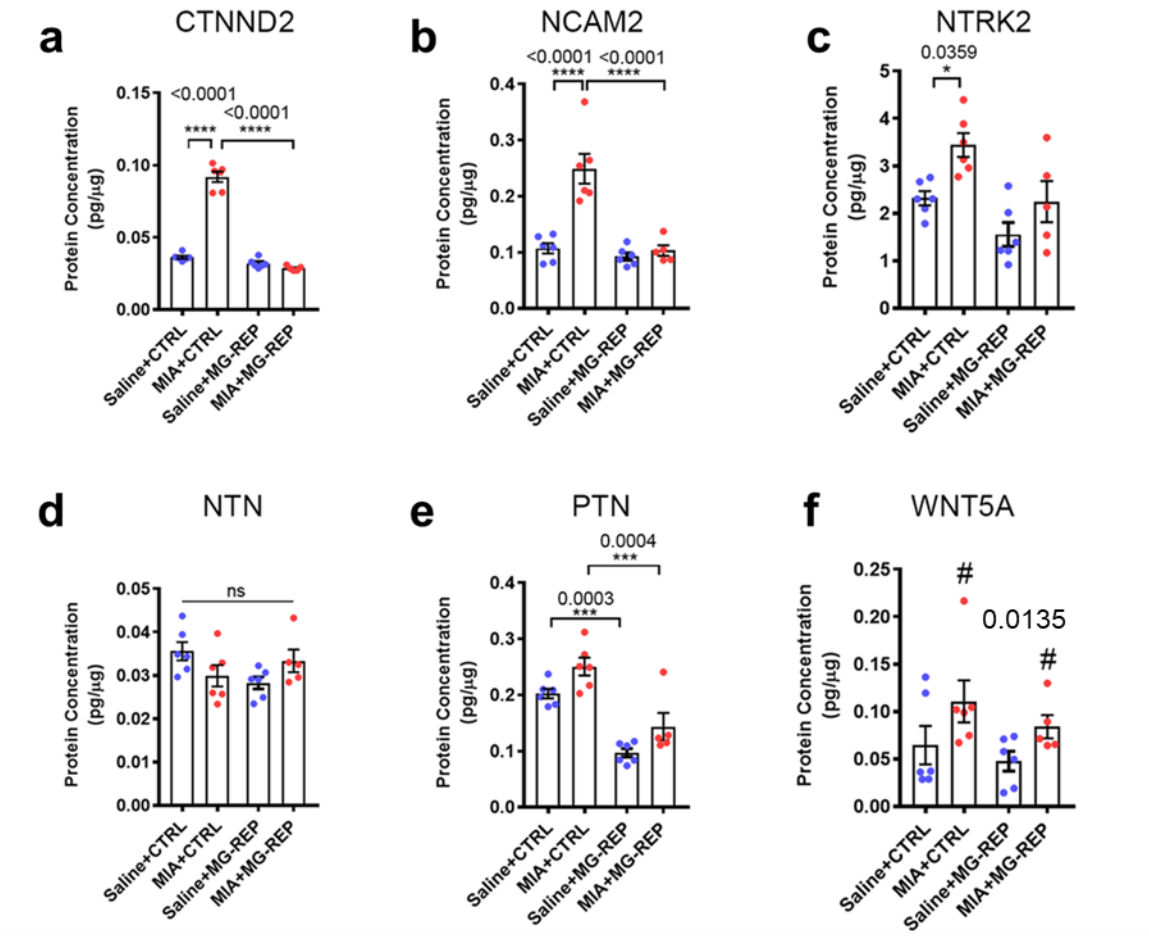


Figure 4.4 Protein level verification of selected neuritogenic molecules using ELISA

Protein expression of CTNND2 **(a),** NCAM2 (**b**), NTRK2 (**c**), NTN (**d**), PTN (**e**) and WNT5A (**f**) in acutely isolated CD11b+ microglia by ELISA. n = (6/4, 6/3, 5/3, 6/3) female mice/litters for P60 Saline + CTRL, MIA + CTRL, Saline + MG-REP and MIA + MG-REP. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 as determined by Two-way ANOVA (alpha = 0.05) with Tukey's post-hoc analysis. WNT5a tested by nonparametric Friedman's Two-way ANOVA due to non-normal distribution. Graphs indicate mean ± SEM. Data collection by H. Yeh and T. Ikezu. Analysis and figure by H. Yeh and T. Ikezu. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

In the early postnatal stage, microglia are essential in supporting survival of layer V pyramidal neurons (Ueno et al. 2013). Dysregulated deep cortical layer pyramidal

neurons in mPFC have been implicated in aberrant neuronal circuitry and synchrony in MIA (Dickerson, Wolff, and Bilkey 2010). To determine MIA affect on neuronal intrinsic properties and activity, we performed whole-cell patch-clamp recordings of layer V pyramidal neurons in the prelimbic mPFC area of P60 male mice (**Figure 4.5a**). Layer V neurons were classified into intrinsically bursting (IB) or regular spiking (RS) based on spiking patterns as described in Chapter 2: Methods (**Figure 4.5b**). IB neurons comprise a smaller subset (10–20%) of layer V pyramidal neuronal with projections to subcortical structures, including striatum and thalamus, whereas RS neurons project to cortex or striatum (Jacob et al. 2012; Kawaguchi 2017). MIA had no significant effect on the RS neuronal electrophysiological properties. But, we found MIA significantly increased the input resistance (Rn) of IB neurons in MIA+CTRL compared to Saline+CTRL offspring, in which this significant difference was not observed between MIA+MG-REP and Saline + CTRL offspring (**Figure 4.5c-d**). IB neurons from MIA+CTRL offspring exhibited significantly prolonged action potential (AP) kinetics (**Figure 4.5e**), including increased AP duration (**Figure 4.5f**), AP rise time (**Figure 4.5g**), and prolonged AP fall time when compared to Saline + CTRL (**Figure 4.5h**). These kinetic changes were normalized by microglia repopulation treatment, as MIA+MG-REP did not differ from Saline + CTRL neurons except in AP rise time (**Figure 4.5f–h**). In addition, microglia repopulation treatment increased input resistance, AP duration, AP rise time, and AP fall time in Saline + MG-REP neurons compared with Saline + CTRL neurons. Further examination may be required to determine whether microglia

repopulation can have sustained and long-term effects on neuronal excitability in saline offspring compared to MIA offspring.

We then recorded spontaneous excitatory postsynaptic current events (sEPSCs) in IB neurons. Compared to Saline + CTRL neurons, MIA significantly decreased the mean sEPSC amplitude of MIA+CTRL neurons, which was not reversed by microglia repopulation treatment (**Figure 4.5i, j**). Furthermore, sEPSCs amplitude in Saline + MG-REP neurons was significantly reduced compared to Saline + CTRL neurons, suggesting functional changes to neuronal activity due to microglia repopulation. MG-REP-induced changes in Saline offspring can be explained by reduction in homeostatic gene expression in repopulated microglia in SAL+MG-REP offspring, such as reduced expression of CX3CR1 that can scupt and refine neuronal synapses. CX3CR1-deficiency reduced the amplitude of EPSC in CA1 hippocampal synapses (Basilico et al. 2019). Induction of DAM/MGnD microglial phenotype can be detected in saline repopulated microglia but not in MIA repopulated microglia, which suggests microglia repopulation treatment may specifically impact glutamatergic synapses maintenance in saline offspring. In summary, IB, but not RS, layer V pyramidal neurons in MIA offspring showed functionally distinct alterations in intrinsic and synaptic response properties, including prolonged AP kinetics and reduced sEPSCs amplitude, in which the effects were partially reversed by microglial repopulation treatment.

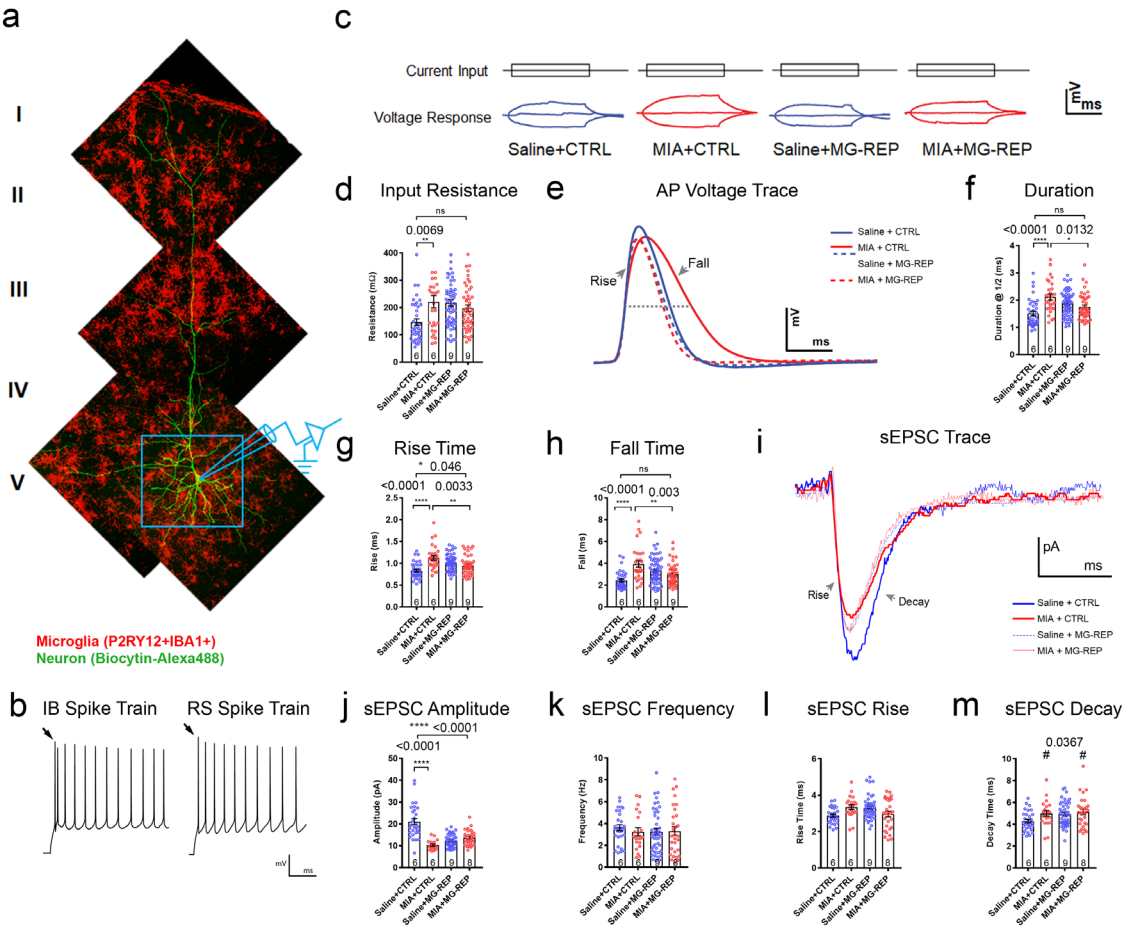

a
I
II
III
IV
V
Microglia (P2RY12+IBA1+)
Neuron (Biocytin-Alexa488)
c
Current Input
Voltage Response
Saline+CTRL
MIA+CTRL
Saline+MG-REP
MIA+MG-REP
d
Input Resistance
Resistance (mΩ)
0.0069
e
AP Voltage Trace
Rise
Fall
Saline + CTRL
MIA + CTRL
Saline + MG-REP
MIA + MG-REP
f
Duration
Duration @ 1/2 (ms)
<0.0001
0.0132
g
Rise Time
Rise (ms)
0.046
<0.0001
0.0033
h
Fall Time
Fall (ms)
<0.0001
0.003
i
sEPSC Trace
Rise
Decay
pA
ms
b
IB Spike Train
RS Spike Train
j
sEPSC Amplitude
Amplitude (pA)
<0.0001
<0.0001
k
sEPSC Frequency
Frequency (Hz)
l
sEPSC Rise
Rise Time (ms)
m
sEPSC Decay
Decay Time (ms)
0.0367

Figure 4.5 MIA induced hyper-excitability in layer V IB neurons, which can be ameliorated by microglial repopulation treatment.

a) Merged confocal z-stacks showing biocytin-filled neuron (green) suing whole-cell patch-clamp and P2RY12+IBA1+ microglia (red) in mPFC of male offspring. **b)** Representative repetitive AP firing responses to +120 pA current step of intrinsically bursting (IB) and regular spiking (RS) layer V pyramidal neuron. Note in the IB cell: the initial AP doublet (arrow, left), versus the RS cell: initial single AP (arrow, right). Scale bar: y-axis = 20 mV, x-axis = 250 ms. **c)** Voltage responses (bottom traces) to 200 ms depolarizing (+20 pA) and hyperpolarizing (−20 pA) current steps (top traces). Scale bar: y-axis = 4 mV, x-axis = 100 ms. **d–h)**: Intrinsic properties of layer V IB neurons: **d**) Mean input resistance (Rn). **e**) Representative traces of AP from layer V pyramidal neurons of Saline + CTRL (blue), MIA + CTRL (red), Saline + MG-REP (blue dotted) and MIA + MG-REP (red dotted) offspring. Scale bar: y-axis = 20 mV, x-axis = 1 ms. **f)** Mean AP duration. **g)** AP rise time. **h**) AP fall time. Number per treatment group: n = (38/6/4, 30/6/4, 60/9/6, 45/9/7) cells/male mice/litters for (Saline + CTRL, MIA + CTRL, Saline + MG-REP, MIA + MG-REP). **i)** Representative waveforms of averaged sEPSC in Saline + CTRL (blue), MIA + CTRL (red), Saline + MG-REP (blue dotted), and MIA + MG-REP (red dotted) layer V neurons. Scale bar: y-axis = 4pA, x-axis = 10 ms. **j)** sEPSC amplitude. **k)** sEPSC frequency. **l)** sEPSC rise time. **m)** sEPSC decay time. Number per group: n = (29/6/4, 20/6/4, 46/9/6, 30/8/6) cells/male mice/litters. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$, ns denotes no significance; determined by Two-way ANOVA (alpha = 0.05) and Tukey's post-hoc analysis. Graphs indicate mean ± SEM. Data collection by S. Sivakumaran, C. Holland, T. Guillamon-Vivancos, M. Medalla, J. Luebke. Analysis by S. Sivakumaran, H. Yeh, K. Ngo and A. Mei. Figure by H. Yeh and J. Luebke. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

Neuronal plasticity and brain circuitry can be mediated by changes in dendritic spine density and morphology. Our results reveal MIA-induced aberrant microglial expression of cellular protrusion and neuritogenic factors, suggesting dendritic spine alterations in MIA mice. Therefore, we determined the effects of MIA and microglial repopulation on dendritic spine density and morphology of biocytin-filled and electrophysiologically characterized layer V IB pyramidal neurons in P60 male offspring as previously described (Basu et al. 2019) (**Figure 4.6**). MIA significantly increased total

and filopodia spine densities but did not affect stubby or mushroom spine densities (**Figure 4.6a-f** and **Figure 4.7a**). Microglia repopulation normalized the MIA-induced differences in total and filopodia spine density between Saline and MIA offspring and had no significant effect on Saline offspring (**Figure 4.6b, f**). Spine dysgenesis and altered synaptic pruning are observed in neurodevelopmental disorders, including increased spine density reported in MIA offspring (Soumiya, Fukumitsu, and Furukawa 2011; Fernández de Cossío et al. 2017). However, contrary to our findings, Coiro et al. found decreased spine density in layer V pyramidal neurons in the somatosensory cortex (Coiro et al. 2015). Different MIA induction protocols may explain this discrepancy since Coiro et al. induced MIA at later timepoint E12.5, targeting a distinct microglial population, and may have a differential effect on brain development (Coiro et al. 2015; De et al. 2018; Ginhoux et al. 2013).

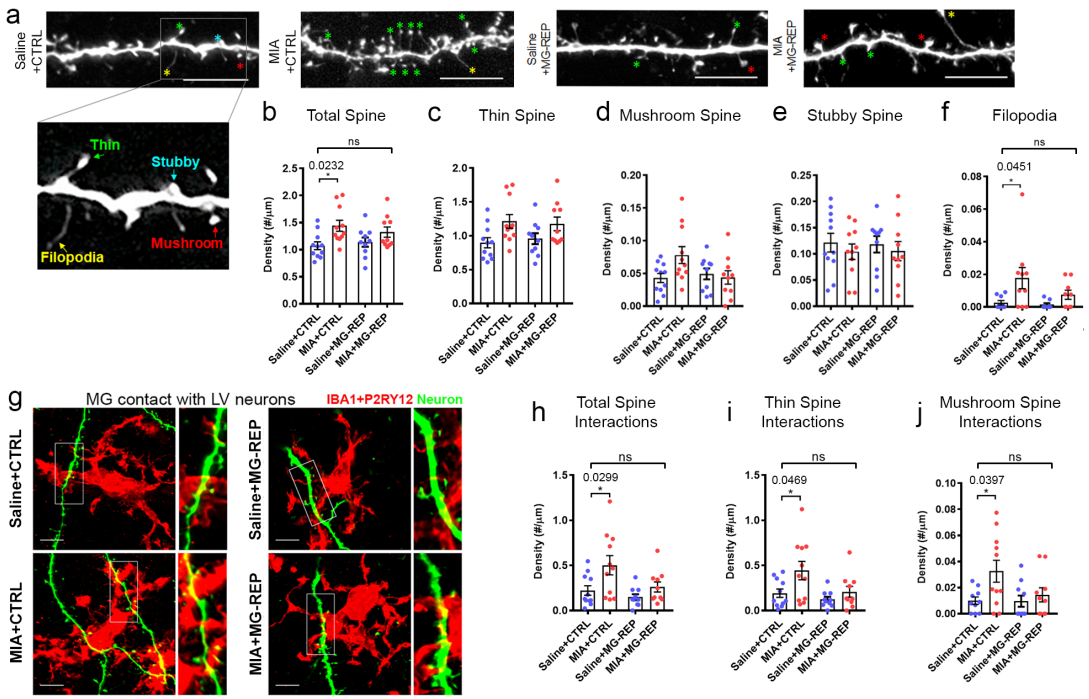


Figure 4.6. Microglia repopulation treatment alleviates MIA-induced upregulation of basal dendritic spine density and microglia-neuron interactions.

a) Representative images of biocytin-filled layer V pyramidal neurons in male mice. Scale bar = 7 μm. Asterisks mark each spine subtype: filopodia (yellow), mushroom (red), stubby (blue), and thin (green) spines. Spine density of layer V IB cells: Total (**b**), thin (**c**), mushroom (**d**), stubby (**e**), and filopodia **(f)** dendritic spine densities. **(g)** Representative images of microglia (red) interacting with dendritic spines (green) of IB neurons. Scale bar = 4 μm. **h-j)** Number of microglial interactions with total, (**h**), thin **(i)** and mushroom spines (**j**). n = (11/8/5/3, 11/8/5/4, 10/8/5/3, 10/4/3/3), dendrites/cells/male mice/litters for (Saline + CTRL, MIA + CTRL, Saline + MG-REP, MIA + MG-REP). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, ns denotes no significance; determined by Two-way ANOVA (alpha = 0.05) and Tukey's post-hoc. Graphs indicate mean ± SEM. Data collection by M. Woodbury, H. Yeh, S. Ikezu, A. Yoshii-Kitahara, M. Medalla, J. Luebke, S. Sivakumaran. Analysis by M. Woodbury, H. Yeh, and Z. Ruan. Figure by H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

Microglia plays a critical role in shaping neuronal development and mediating synaptic plasticity (Tremblay, Lowery, and Majewska 2010). Direct microglial contacts with dendritic shafts induce filopodia formation (Weinhard et al. 2018; Miyamoto et al. 2016). Therefore, we hypothesized that altered microglia-neuron interaction contributes to the dendritic spine changes observed in MIA+CTRL mice. We quantified microglial interactions with biocytin-filled basal dendritic spines of layer V IB pyramidal neurons from adult P60 male offspring (**Figure 4.6g** and **Figure 4.7b–e**). MIA significantly increased microglial interactions with total, thin, and mushroom spines in MIA + CTRL compared to Saline + CTRL offspring and microglial repopulation (MIA + MG-REP) negated this effect (**Figure 4.6h**–**j**). Microglial interactions with dendritic spines were divided into three subtypes based on the 3D distance between microglial processes and dendritic spines (**Figure 4.7f, g**). All microglia–spine interactions were increased in

MIA + CTRL compared with the Saline + CTRL offspring, but there were no significant differences between Saline + CTRL and MIA + MG-REP offspring (**Figure 4.7h-j**). These results demonstrate that MIA can alter microglia-neuron interactions and possibly mediate dendritic spine growth or maintenance in adulthood.

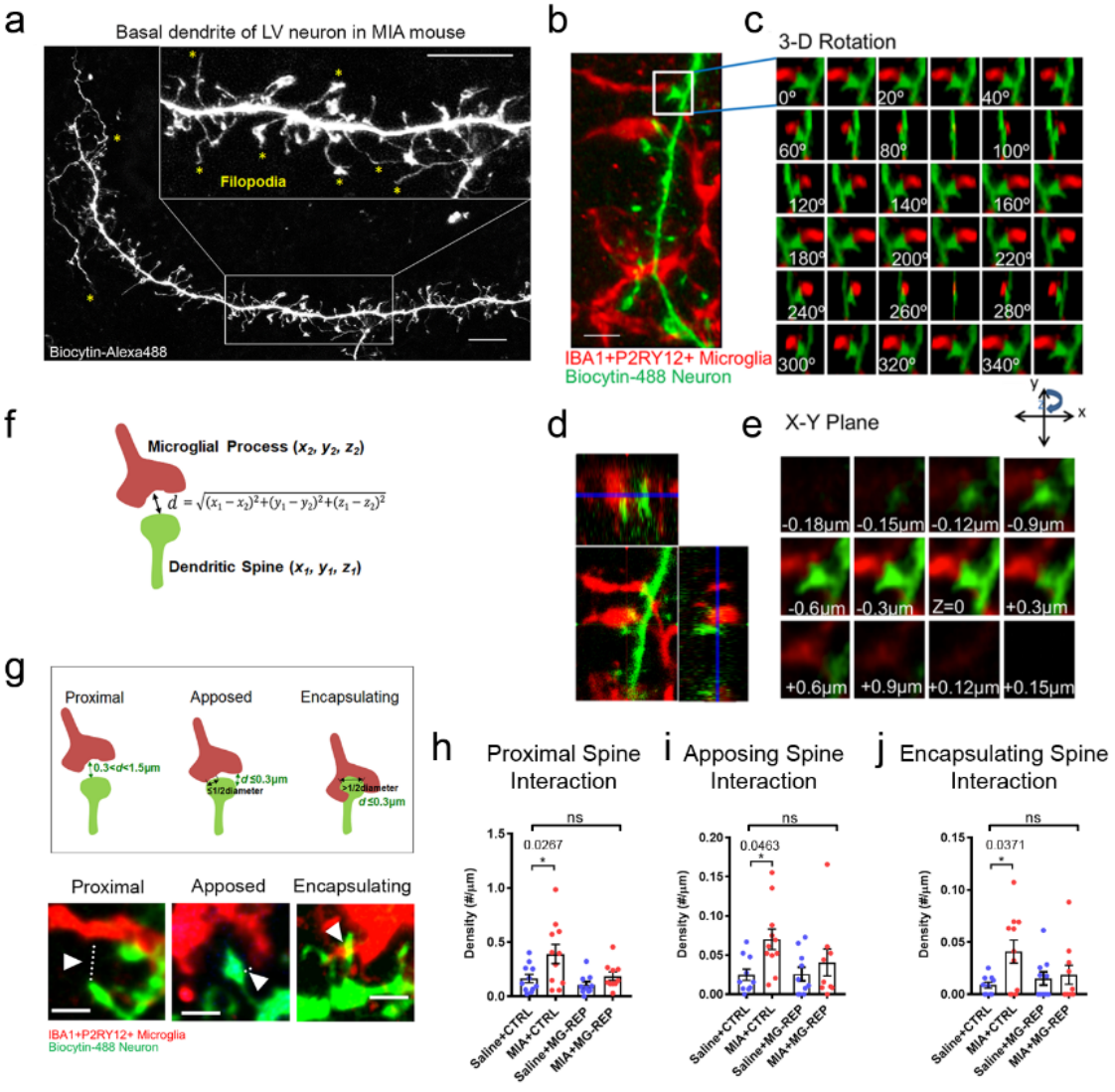


Figure 4.7 Confocal laser scanning microscopic imaging and quantification of microglial interaction with synapses in Layer V mPFC of P60 male mice.

a), A representative image of a basal dendrite of a biocytin-filled Layer V pyramidal IB cells of P60 MIA+CTRL male mouse with multiple filopodia formation in basal dendrites (yellow asterisk). Scale bar = 7 µm. **b-e,** Analysis of microglial interaction with dendritic spines. Representative 40× confocal images of microglia interaction with neurons: **b),** Basal dendrite of layer V pyramidal neuron (green) and P2RY12/IBA1+ microglia (red). White box denotes area shown in b. **c),** Complete 3D rotation of spine-microglia interaction for quantification, at 10-degree increments. **d),** Detail of spine microglia interactions around the white box inset in b, shown in xy, yz, and xz maximum projection images. **e),** Individual xy plane optical slices, imaged at z-increments of 0.3 µm. **f),** Formula for the calculation of distance between the microglia process and dendritic spine. The distance shorter than 1.5 µm is defined as microglial interaction with dendritic spines. **g),** Diagram (top) of microglia-spine interaction types and representative confocal images (bottom) of microglial processes (red) interacting with the dendritic spines of biocytin-filled layer V IB neurons (green). Arrowheads indicate distance between the two processes. **h-j,** Proximal (**h**), apposition (**i**) and encapsulating (**j**) spine-interactions normalized to basal dendrite length. n = (11/8/5/3, 11/8/5/4, 10/8/5/3, 10/4/3/3), dendrites/cells/male mice/litters for (Saline+CTRL, MIA+CTRL, Saline+MG-REP, MIA+MG-REP). * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$, ns denotes no significance; determined by Two-way ANOVA (alpha = 0.05) and Tukey's post-hoc. Graphs indicate mean ± SEM. Data collection by M. Woodbury, H. Yeh, S. Ikezu, A. Yoshii-Kitahara, M. Medalla, J. Luebke, S. Sivakumaran. Analysis by M. Woodbury, H. Yeh, and Z. Ruan. Figure by H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

To validate the effect of MIA microglia on spine formations, we analyzed the dendritic spine density of primary mouse cortical neurons co-cultured with FACS-purified E17.5 microglia (**Figure 4.8a**). Isolation of E17.5 microglia from Saline or MIA embryos enabled characterization at the critical time point with most enrichment of neuritogenic genes in MIA microglia. At days in vitro 19 (DIV19), MIA microglia co-culture significantly increased the proportion of filopodia spines when compared to saline microglia co-culture (**Figure 4.8b**), consistent with our *in vivo* findings of enhanced filopodia formation in MIA+CTRL offspring. These findings imply morphological

changes in layer V pyramidal neurons can be driven by MIA microglial secretory factors that promote neuritogenesis.

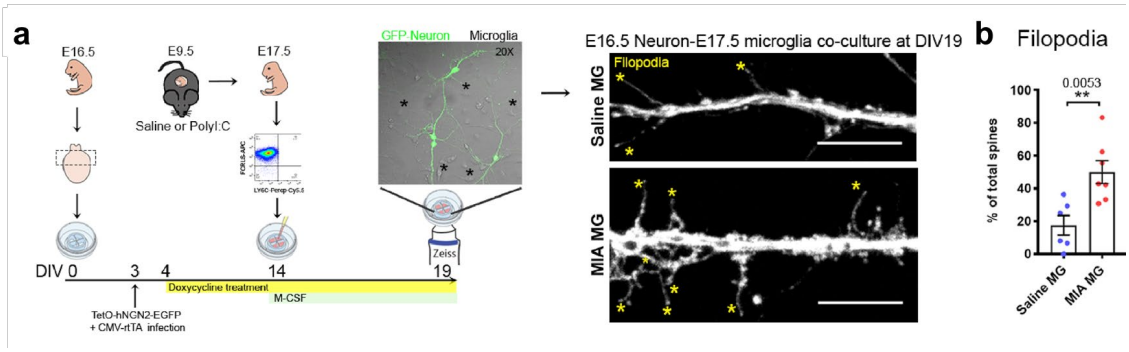


Figure 4.8. Confocal microscopic live imaging analysis of effect of MIA microglia on dendritic spine formation in murine primary cultured cortical neurons.

a), GFP-labeled E16.5 primary cultured cortical murine neurons at DIV19 was co-cultured with FACS-purified E17.5 murine microglia from saline (Saline MG) or MIA offspring (MIA MG). n=6-7 dendrites per group from pooled E17.5 embryos/litter. Representative images show increased filopodia in neuronal dendrites co-cultured with MIA microglia. **b)** The total % of filopodia spines is increased when co-cultured with MIA MG. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001, ns denotes no significance; determined by *t*-test (alpha = 0.05). Graphs indicate mean ±SEM. Data collection by H. Yeh and Y. You. Analysis and figure by H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

Finally, to determine the association between molecular and physiological changes, pairwise Spearman's correlation analyses were performed to analyze the strength of association between morphological, neurophysiological properties, and corresponding microglial-neuron interactions (**Figure 4.9**). The number of microglial branches had a significant positive association with microglial-spine interactions (total and thin spines) and mushroom spine density. In addition, increased microglial processes was positively associated with enhanced membrane resistance (Rn) and prolonged

kinetics of APs (duration, rise, fall), and excitatory postsynaptic activity (sEPSC rise and decay), reflective of postsynaptic neuronal activity. Lastly, microglial interaction with dendritic spines (total, thin, and mushroom spines) was positively correlated with action potentials kinetics (duration, rise, fall), suggesting MIA can alter microglial-neuron interactions and possibly contribute to neuronal intrinsic and AP firing pattern changes. Collectively, this study demonstrates that MIA induces aberrant microglial morphological changes, enhances microglia-spine interactions and synaptogenesis, neuronal hyperexcitability, and impairs social behavior, which were partially alleviated by microglial repopulation treatment (**Figure 4.10**). Our Spearman correlation analysis reveal a synergistic positive correlation of microglial branching with neuronal synaptic interactions, intrinsic AP kinetics, and EPSC properties of layer V IB neurons.

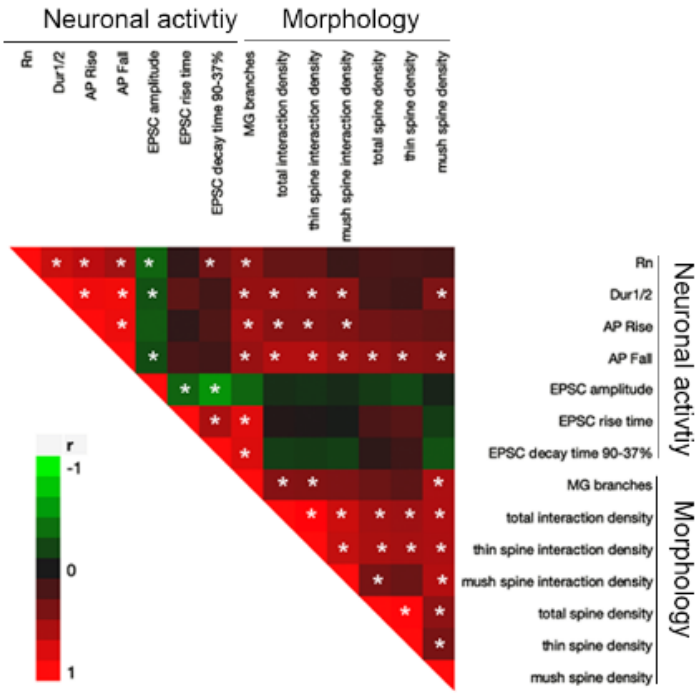


Figure 4.9. Spearman's correlation analysis of neuronal excitability and morphological characterization from within-cell comparisons.

The properties of neuronal excitability and AP firing kinetics from IB neurons consist of Rn, Dur, AP rise, AP fall, sEPSC amplitude, sEPSC rise time, and sEPSC decay time. Morphogly characterization include microglia branching (at 9th order), total spine microglia-neuron interaction density, thin spine interaction, mushroom spine interaction, total spine density, thin spine density and mushroom spine density. Rho values for correlation: red (1) to green (−1). * denotes $p < 0.05$ as determined by Pearson's correlation. Data collection by M. Woodbury, H. Yeh, S. Ikezu, A. Yoshii-Kitahara, M. Medalla, J. Luebke, S. Sivakumaran. Analysis by M. Woodbury, H. Yeh. Figure by M. Woodbury and H. Yeh. Data and figure published in Ikezu, Yeh, Delpech, Woodbury et al. *Molecular Psychiatry* (2020).

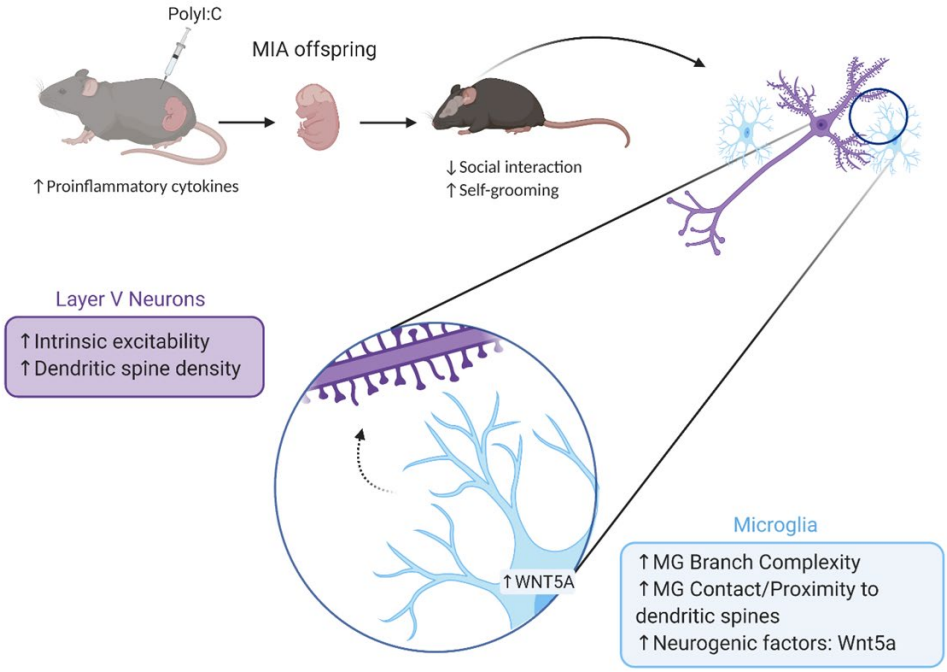


Figure 4.10. Schematic of MIA effect on behavior, neurons and microglial.

Induction of MIA by poly (I:C) led to increased cytokine production during prenatal development. This altered MIA offspring development and behavior outcome consisting of reduced social interaction and increased repetitive behavior. Whole-cell patch clamp-recordings revealed MIA increased intrinsic excitability associated increased spine density in layer V neurons. MIA increased neuron-microglia contact and hyper-ramification of microglia. Transcriptional analysis and validation experiments confirmed upregulation of neuritogenic molecules that could be secreted by microglia that induces spine changes and neuronal excitability. Figure by H. Yeh.

4.3 Discussion

This study reveals that microglia play a critical role in mediating atypical neuronal development and behaviors induced by MIA. Establishing the causal relationship between prenatal neuroimmune abnormalities and neuronal developmental defects has been challenging. Our study revealed elevated expression of molecules that facilitate neurite outgrowth at the mRNA level by transcriptomic analysis, *in situ* hybridization, qPCR, and at the protein level by ELISA in MIA microglia compared to Saline microglia. Transcription and translation changes of specific synaptic genes could be a novel convergence point for MIA-induced and genetic neurodevelopmental diseases. Synaptic and neuritogenic genes in microglia are enriched in ramified microglia, suggesting that upregulation of these genes may correspond with specific roles of microglia during brain development (Parakalan et al. 2012). Microglia can secrete neurotrophic and neuritogenic molecules that alter microglia-neuron interactions and modulate neuronal physiology. In embryonic and adult microglia, MIA increased the expression of neuritogenic/neurogenic genes, including *Wnt5a* and *Ncam2*. This further

suggests that transient immune activation during the prenatal period may have a long-lasting impact on microglia phenotype and alter microglia-neuron interactions.

Microglia have been shown to express neuritogenic genes, including PSD95, which are developmentally regulated and modulated by inflammation status (Krishnan et al. 2017). Inflammation disrupted the developmental pattern of transient microglial synthesis of PSD95 in both mouse and human brains (Krishnan et al. 2017). *Ncam2* is expressed in both neurons and microglia, and the release of NCAM2, an adhesion molecule critical for synaptogenesis, can trigger intracellular calcium elevation and subsequent actin recruitment and filopodia formation (Sheng, Leshchyns'ka, and Sytnyk 2015; Zhang et al. 2014)). Microglia release of synaptogenic/neuritogenic molecules instead of neurons can result in a more targeted approach of modulating specific synapses and circuitry since microglia are highly motile and attracted to active synapses (Badimon et al. 2020). The upregulation of neuritogenic genes, including microglial NCAM2, may contribute to excessive neuritogenesis and increased neuronal excitability in specific neuronal circuits. We identified increased excitability in IB pyramidal neurons but no MIA-induced changes in RS layer V pyramidal neurons. Here, we systematically characterized the role of microglia in MIA model and assessed the effect of microglial repopulation treatment using behavioral, electrophysiological, cellular, and molecular approaches. We show MIA has a long-lasting impact on microglial transcriptome and microglia–synapse interactions, which also corresponded to synaptic defects, including increased immature spine density and behavior changes.

Mutations in ASD-related genes such as *FMR1*, *PTEN, EIF4E*, and *CYFIP*, correspond to elevated transcription and protein translation, increased filopodia-like protrusions, and altered synaptic strength (Bourgeron et al. 2015; Penzes et al. 2011). Among the MIA-enriched cellular protrusion/neuritogenic molecules in our MIA microglial transcriptome, *Ctnnd2*, *Enc1*, *Brsk2*, *Ntrk2*, *Ntrk3*, and *Ryr2* are genes targeted by FMRP that is deficient in Fragile X syndrome (Darnell et al 2011, Brown et al. 2001). Microglial enrichment of genes associated with ASD and Fragile X syndrome suggests MIA microglia can alter neuronal circuit formation and abnormal behaviors in neurodevelopmental disorders.

Other studies have shown changes in microglia phenotype by MIA. Previous work showed that MIA induced a decrease in microglial tip velocity, suggesting MIA increases brief pauses during brain surveillance (Ozaki et al. 2020). This motility may be regulated by trophic signals (Mosser et al. 2017). This MIA-increased microglial motility suggests MIA-enhanced microglial-neuron interactions can mediate synapse formation and be further examined with *in vivo* imaging in future studies. A more recent *in vitro* study corroborated our findings that MIA can alter neuronal activity. Spontaneous network activity in primary hippocampal neuronal cultures isolated from MIA offspring at 21 DIV was greater compared to cultures derived from control mice (Wegrzyn et al. 2020). This hyperexcitability was associated with structural changes in synaptic marker expression, including increased PSD95 and VLGUT2 *in vitro* (Wergrzyn et al. 2020).

Based on our study, microglial repopulation by CSF1R inhibition may be a potential therapeutic approach for neurodevelopmental disorders associated with maternal

inflammation. Further examination of peripheral effects of CSF1R inhibition may need to be addressed as well since CSF1R inhibition by PLX5622 is not specific to CNS (Lei et al. 2020). PLX5622 is an improved CSF1R inhibitor compared to PLX3977 with increased brain penetration and increased specificity targeting CSF1R (Spangenberg et al. 2019). In human clinical trials for treating tenosynovial giant cell tumors, adverse effects of PLX3977 include hepatic adverse reactions but show a tolerable safety profile with no late-emerging toxicity (Gelderblom et al. 2021). Chronic stress such as repeated social defeat causes microglia to be sensitized and reactive. Repopulation of microglia attenuated the amplified response to LPS by reducing cytokine response (Weber et al. 2019). CSF1R inhibition-induced microglia repopulation treatment reverses MIA-induced changes, including increased immature spine densities and impaired social interaction in MIA offspring, suggesting that inflammation-induced microglial and synaptic dysfunction may be plastic and reversible. A recent single-cell RNA-seq study of repopulating microglia at 5 days post-withdrawal of CSF1R inhibitor shows that repopulating microglia is distinct from the original microglia and upregulation of genes associated with immature microglia at day 5 (Huang et al. 2018). This transient upregulation of immature genes indicates that repopulation may facilitate the functional correction of MIA microglia, possibly through epigenetic mechanisms. CSF1R inhibition and repopulation treatment downregulates MIA-induced overexpression of microglial expression level of cellular protrusion/neuritogenic factors and restores microglia homeostatic immune profiles. Aberrant neuronal support by exacerbated secretion of

those factors from MIA microglia could induce hyperconnectivity and, in turn, disrupt the precise programming and wiring of neural networks during neurodevelopment.

CHAPTER FIVE

Role of neurogenic Wnt5a in microglia on neurodevelopment

5.1 Introduction

Neuroinflammation may be a key mechanism in accelerating pathology in neurological disorders. Microglia are critical contributors to inflammation in the brain. In addition, microglia play an essential role in mediating neuronal development. One of the regulators we hypothesize to be important in microglia-neuron communication is Wingless-related MMTV integration site 5a (Wnt5a). Wnt5a is a known mediator of immune responses to inflammation and neurodegenerative disorders such as Alzheimer's disease (Halleskog et al. 2011; Yang and Zhang 2020). Studies have shown that the neurotrophic factor Wnt5a plays an indispensable part in neurodevelopment (Chen et al. 2017; Blakely et al. 2011). Previously, we found maternal immune activation (MIA) significantly upregulates microglial expression of Wnt5a that was strongly associated with enhanced growth of filopodia dendritic spines (Ikezu et al. 2020). Investigating how microglia can affect synaptic remodeling may explain how synapses become altered under pathological conditions involving microglial dysregulation. We hypothesized that microglial could secrete WNT5a and regulate dendritic spine development and maintenance, and neural circuitry. Therefore, altered excessive microglial Wnt5a due to inflammation can lead to abnormal neuronal activity.

Disruption of the canonical Wnt pathway leads to altered neuronal migration, disturbed circuit development, and behavioral deficits (Bocchi et al. 2017). Wnt signaling pathways can be classified into canonical and non-canonical pathways. Recent studies

reveal the complexity of Wnt signaling pathways due to the number of intracellular cascades. The Wnt family is composed of 19 Wnts in mammals and is crucial in stem cell differentiation and differentiation during embryonic development (Kikuchi et al. 2012). The majority of Wnt ligands including Wnt1, Wnt3a, Wnt7a/b, and Wnt8 mediate the canonical Wnt/β-catenin pathway, whereas others Wnt4, Wnt5a, Wnt9b and Wnt11 mediate the non-canonical Wnt/Ca^{2+} or Wnt/PCP/JNK, also known as the Wnt/planar cell polarity pathway (PCP), pathways independent of β-catenin (Oliva, Vargas, and Inestrosa 2013). Wnt pathways could be activated by Wnt ligands binding to the 15 different receptors and co-receptors dependent on cellular context and specificity (Oliva et al. 2013).

WNT5a, a secreted glycoprotein, acts primarily through a non-canonical Wnt pathway depending on receptor context (Mikels and Nusse 2006). WNT5a binds to receptor Frizzleds (Fzds) and ROR2, a receptor tyrosine kinase, and activates the JNK and TGFb signaling pathways (Bian et al. 2015; Nye et al. 2020; Shao et al. 2016). Wnt5a activation can increase neurite branching complexity in CA1 pyramidal neurons or GABAergic interneurons and stimulate PSD95 or GABAA receptor insertion on neuronal surface membranes through CaMKII pathway (Cuitino et al. 2010; Paina et al. 2011). Previous studies have shown that Wnt5a plays a critical role in regulating developmental axon and dendrite outgrowth and synapse formation in neurons (Halleskog et al. 2011; Chen et al. 2017). WNT5a decreases the number of presynaptic inputs in immature hippocampal neurons *in vitro,* suggesting a possible antagonistic effect of WNT5a on canonical Wnt signaling (Davis, Zou, and Ghosh 2008). Other

studies show that WNT5a activates the JNK pathway and acts primarily on post-synaptic sites of differentiated hippocampal neurons in vitro and in vivo, increasing spine density and clustering of PSD95 in dendritic spines (Chen et al. 2017; Varela-Nallar et al. 2010; Farías et al. 2009). In hippocampal neurons, WNT5a stimulates calcium and cytoskeletal-mediated signaling pathways for transcription of the NMDA receptor subunit GluN1, suggesting WNT5a acts primarily through post-synaptic mechanisms (Halleskog et al. 2011; Chen et al. 2017). Further investigation is needed to determine if abnormal microglial Wnt5a expression can affect brain circuitry. Here, we investigated the effect of silencing Wnt5a in microglia on neuron development and physiology *in vitro*.

5.2 Methods

Genome-wide microarray profiling of microglia and neural stem cells

Following MACS isolation using CD11b+ magnetic beads (Miltenyi) according to manufacturer's protocol as previously described (Ikezu et al. 2020), primary mouse microglia were cultured overnight in DMEM supplemented with murine 20ng/ml M-CSF recombinant (R&D) and media supernatant were collected the following day for conditioned media. Microglia lysate were digested with QIAzol (79306, Qiagen) and RNA was isolated using the miRNeasy Mini Kit (217004, Qiagen). For neural stem cell gene expression analysis, neural stem cells were plated for 24 h with conditioned media from primary microglia culture or control neurobasal media and collected using Qiazol lysis reagent and total mRNA extracted using miRNeasy Kit (Qiagen). Microarray kits used include the Affymetrix GeneChip Mouse Exon 1.0 ST Array (900818, Affymetrix)

for microglia expression profiling and Affymetrix Mouse Gene 2.0 ST array (902118. Affymetrix) for neural stem cell analysis. Microarray data were generated and analyzed by Boston University School of Medicine Microarray Core facility. Microarray data were uploaded to the NCBI GEO repository (GSE49329).

Neural stem cell gene expression data was analyzed using moderated t-test. Ingenuity pathway analysis (IPA) identified the most highly- upregulated canonical pathways in microglia co-cultured with or without neural stem cells. To identify biologicals pathways unique to neural stem cells cultured with or without microglia-conditioned media, expression data files were analyzed with IPA (Qiagen) or Panther 16.0 http://pantherdb.org/. IPA core analysis based on log2 fold change for neural stem cells with microglial conditioned media group versus control NSCs group. Statistical analysis on microarray data was analyzed using moderated t-test in the case of pairwise comparisons with help from Boston University School of Medicine's Microarray Core facility,

Thy1-YFP neuronal culture for DIV17-22 live imaging and Multi-electrode Array (MEA) recordings

Primary mouse neurons were cultured as previously described(You et al., 2020). Embryonic cortices were dissected in Hibernate-E medium (Gibco Invitrogen) on ice, minced into 1 mm^3 pieces, and dissociated into single cells by using 0.25% trypsin-EDTA solution (Gibco Invitrogen) at 15 min at 37°C and gentle pipetting. Primary E16.5 neurons isolated from Thy1-YFP (Stock# 003782, Jackson Laboratory, (Feng et al. 2000) embryos were plated onto PDL-coated 24-well glass-bottom plates (CellVis) and 4 × 4

microelectrodes MEA 24-well plate (Axion Biosystems) with neuronal density of 2.5 × 10E5 cells/well using 20% FBS DMEM (Gibco Invitrogen). Culture medium was replaced with complete neurobasal medium (Neurobasal, 2% B-27, 2 mM Glutamax, Gibco Invitrogen). 50% of media was changed every 3-4 days.

Following 5 min equilibration, MEA recordings began after MEA plates were placed in the Maestro MEA system (Axion Biosystems) at 37°C and 5% CO2 for 15 min recording using AxIS Navigator (Axion Biosystems) with 200–3000 Hz cut-off frequencies. Neuronal spikes were detected using adaptive threshold. Neuronal bursts were automatically calculated using Inter-Spike Interval (ISI) threshold algorithm. Network bursts were computed using an Envelope algorithm with the default threshold. MEA spike data were analyzed by using Axion Biosystems Neural Metrics Tool.

siRNA silencing of WNT5a in microglia and qPCR validation

Accell siRNA was purchased from Horizon Discovery (USA). Lyophilized siRNA was reconstituted in RNAse-free 1x siRNA buffer at a stock concentration of 100uM. Product codes of siRNAs with target transcripts are E-065584-00-0005 (mouse Wnt5a) and K-005000-G1-02 (mouse Control siRNA). siRNA was resuspended in Accell Delivery Media supplemented with M-CSF 20ng/ml (Horizon Discovery) to a final concentration of 1uM Accell siRNA to 50,000 murine microglia. Following isolation of microglia from CD1 E17.5 embryos using CD11B MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol and previously described (Ikezu et al. 2020), microglial cells were plated in 48-well plates with DMEM supplemented with 10%FBS and 25ng/ml M-CSF. At DIV2, cells with incubated with Accell siRNA for additional 3

days and dissociated with 25% Accutase in PBS for qPCR validation and co-culture with neuron at DIV14.

Incucyte live imaging for phagocytosis assay

For phagocytosis assay, primary microglia cells were plated in 48-well. siRNA transfection was performed at DIV2 and incubated for 3 days. After transfection at DIV5, media changed to 2%FBS/DMEM supplemented with 20ng/ml M-CSF (R&D Biotech). 10ug/ml Red pHrodo E Coli bioparticles (Sartorius) were immediately added before imaging, and used as a phagocytic indicator (ref?). Both phase and orange channels were captured for microglial confluence and fluorescence signal of internalized red-pHrodo bioparticles respectively. Images were captured by 20x IncuCyte S3 (Satorius) every 15 minutes for the first 3 hours, then at 1-hour intervals.

Live imaging and analysis at DIV17 and DIV22

At DIV17 and D22, live imaging was captured by confocal microscopy with incubation system at 37C and 5%CO2 (Leica SP8 with Resonance Imaging) using 40x water objective at a resolution of 1024 x 1024. Z-stack images were taken over 15 imaging sessions at 1 min intervals (z step = 0.4um, 8-10um) with adapted focus to maintain z-stack over time, focusing on neuronal dendrites and spines. Images were processed and deconvoluted using Leica Lightning. Dendrite properties were quantified using IMARIS (Bitplane). Spine density was counted from a total dendritic length of ~50 μm using ImageJ software (n = 2-5 neurons per group) at began and end of imaging period. The protrusion rates gained and lost were determined, respectively, as the fraction of spines that appeared and disappeared between two successive frames relative to the

total spine number, as described previously (Cruz-Martín, Crespo, and Portera-Cailliau 2010). Dendritic spine turnover rate was calculated as the total number of spines lost and gained divided by twice the total protrusion number (Cruz-Martín, Crespo, and Portera-Cailliau 2010).

Immunocytochemistry

At DIV23, following the completion of live imaging, cells were fixed by 4% paraformaldehyde for 15 minutes at room temperature. Cells were then washed and permeabilized by 0.1% Triton-X in PBS. Then, cells were blocked for 1 hour using a blocking buffer of 5% NDS in PBS. Primary antibodies Gt-PSD95 (1:500, Abcam), and Gp-VGLUT2 (1:500, Synaptic Systems) were incubated overnight in staining buffer 5%NDS+0.1%Triton-X+0.2%BSA at 4C. The following day, cells were washed with PBS and incubated with secondary antibodies at 1:1000 dilution (Alexa 546 donkey-gt Invitrogen, 647-donkey guinea pig).

Imaging and analysis

Confocal imaging of fixed cells was captured by confocal microscopy (Leica SP8) using a 40x water objective at a resolution of 1024 x 1024, z-step= 0.3 um. All images were deconvoluted and processed using Leica Lightning to enhance resolution and clarity. Following image processing, image analysis was completed by IMARIS (Bitplane) for unbiased and automated quantification of synaptic puncta VGLUT2 and PSD95, and the colocalization by using volume function. The threshold for colocalization was set for 0um distance between VGLUT2 and PSD95.

5.3 Results

Microglia can communicate with neurons by secretory molecules. To determine the effects of microglial secretory molecules on neurodevelopment, genome-wide gene expression analysis was performed on embryonic neural stem cells stimulated with control or microglia conditioned media (**Figure 5.1a**). The enriched genes by microglia conditioned media are associated with neuropeptide signaling pathways, interferon-gamma production, and adenylate cyclase-modulated G-protein coupled receptor signaling pathway, or immune-related response pathways (**Figure 5.1b,** top red). Downregulated genes by microglial conditioned media are associated with pathways involved in translational initiation, telomere maintenance, and mitotic/anaphase transition (**Figure 5.1b,** bottom blue). We identified top canonical pathways influenced by microglia-secreted factors, which include neural differentiation, axonal guidance, and stem cell pluripotency using Ingenuity Pathway Analysis (IPA) (**Table 5.1**). *Wnt* signaling was highly represented in neural stem cells by Wnt/β-catenin pathway, axonal guidance signaling, and stem cell pluripotency. These results suggest the microglial secretome enhanced neural stem cell differentiation.

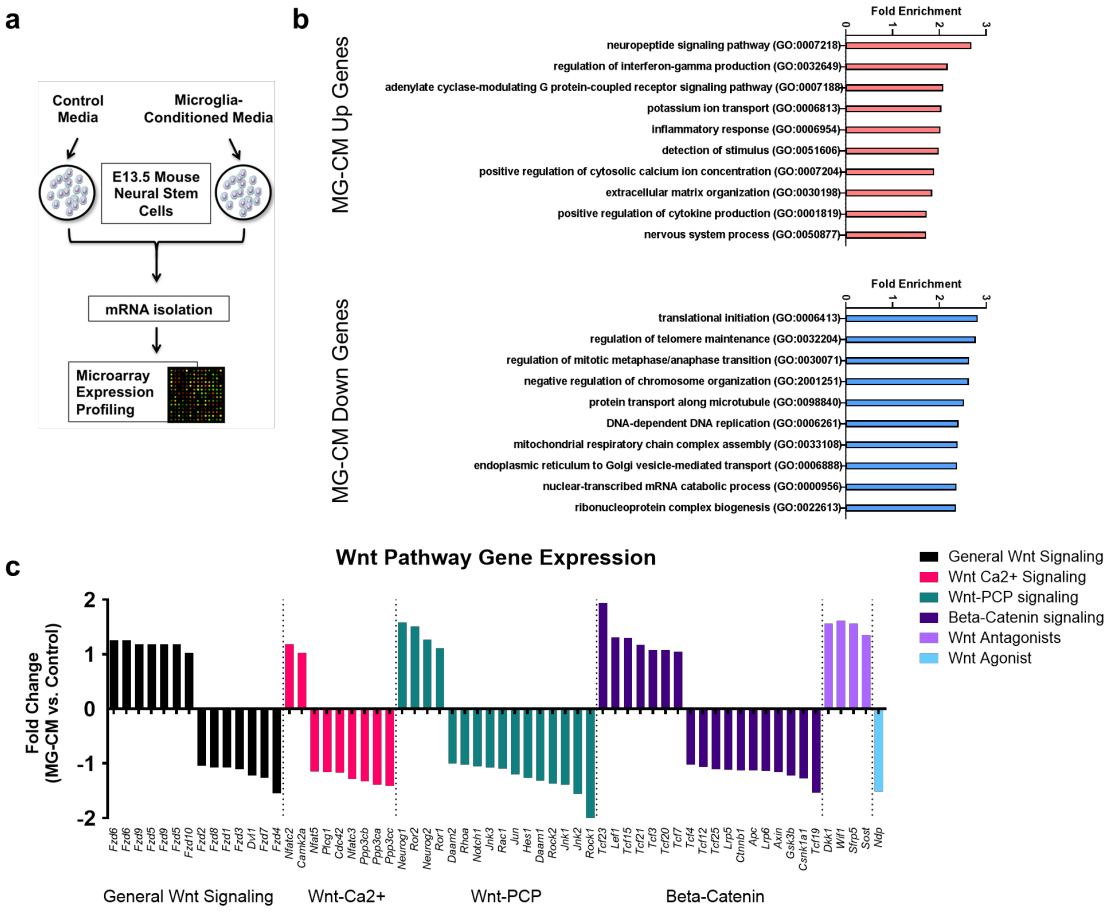


Figure 5.1. Neural stem cells treated with microglia conditioned media show enrichment of genes associated with neuronal maturation

a), Microarray of neural stem cell following 24 h treatment with control or conditioned microglia media. **b)**, Gene ontology analysis of 196 genes increased (top) and 208 decreased (bottom) by microglia conditioned media treatment. (Genes with $p<0.05$, FDR<0.05, and fold change greater than/equal to 2 or less than/equal to -2). **c)**, Relative expression fold change of *Wnt* pathway genes in MG-CM NSCs compared to control NSCs. Several genes are involved in both canonical and non-canonical pathways. Black represents general *Wnt* pathway. Red represents non-canonical Wnt-Ca2+ signaling pathway. Teal represents non-canonical Wnt/planar cell polarity (PCP) signaling pathway. Navy blue represents canonical Wnt/β-catenin pathway. Purple represents Wnt antagonists, whereas light blue represents Wnt agonist Ndp. (Data collection and analysis by K. Ingraham, M. Woodbury, H. Yeh). Figure by H. Yeh and M. Woobury.

Ingenuity Canonical Pathways	-log(p-value)
G-Protein Coupled Receptor Signaling	10.70
Axonal Guidance Signaling	8.31
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	6.43
Nicotinate and Nicotinamide Metabolism	6.02
Inositol Phosphate Metabolism	5.82
Mouse Embryonic Stem Cell Pluripotency	5.60
Signaling by Rho Family GTPases	5.36
cAMP-mediated signaling	5.19
Wnt/β-catenin Signaling	4.99
NGF Signaling	4.93

Table 5.1. Top Ingenuity canonical pathways enriched in neural stem cells with conditioned microglia.

Furthermore, *ROR1s, ROR2,* and several *Wnt* interacting Frizzled receptors were up-regulated in NSCs with microglia conditioned media relative to control media (**Figure 5.1c**). In contrast, several putative *Wnt* intracellular signaling components in this pathway were down-regulated **(Figure 5.1c).** Wnt5a, the best characterized noncanonical Wnt and found to be upregulated by MIA, activates receptor tyrosine kinase ROR1/2-disheveled (Dvl)/ c-jun N-terminal kinase (JNK) pathway (Ho et al. 2012; Ikezu et al. 2020). Both ROR1/2 and WNT5a knockout mice displayed dwarfism and short limbs (Oishi et al. 2003). qRT-PCR confirmed *Ror1* and *Ror2* up-regulation in neural stem cells cultured with microglia-conditioned media (**Figure 5.2a**). Previous studies have shown that inflammation can lead to upregulation of Wnt5a expression in cortical neurons, thereby inducing early neuronal differentiation of cortical neural precursor cells via RhoA/Rho-associated kinase (ROCK) and JNK pathways (Park, Kang, and Han 2018). RhoA/ROCK pathway regulates cytoskeletal and signaling pathways associated with various neuronal functions, including migration, dendrite development, and axonal branching (Fujita and

Yamashita 2014). Thus, we propose that the microglia secretome can promote neural differentiation and dendrite development via WNT activation of ROCK and JNK pathways (**Figure 5.3b**).

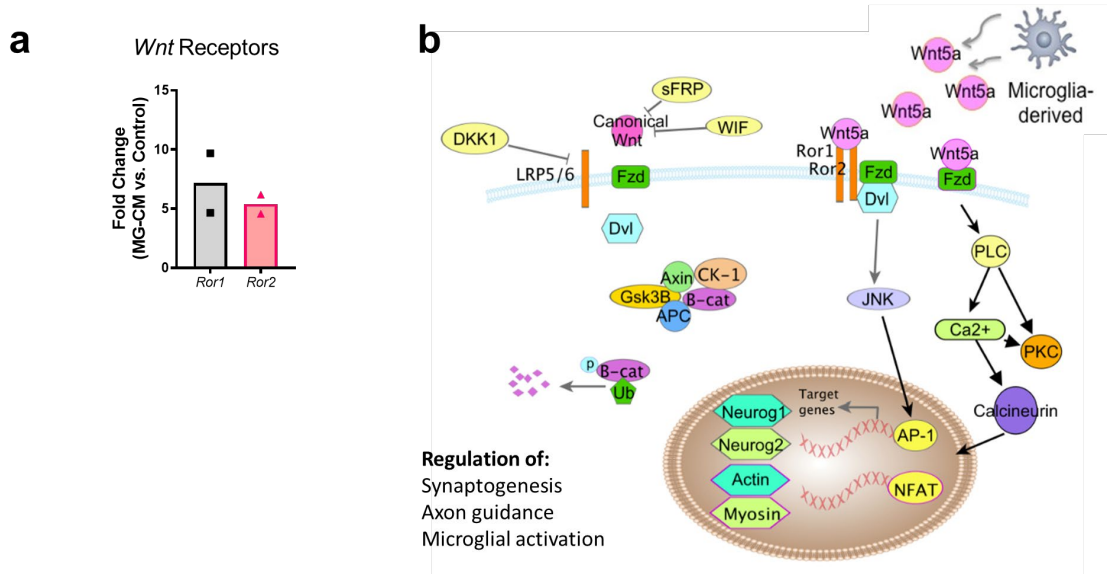


Figure 5.2. Wnt Pathway Gene expression in neural stem cells is significantly altered by microglial signaling factors.

a), RT-PCR confirmation of ROR1 and ROR2 upregulation in NSC cultured with microglia-conditioned media relative to control media. (n=2 replicates). **b),** Proposed Wnt5a signaling pathway in NSC stimulated with microglia-conditioned media. Wnt5a is secreted by microglia and binds to Wnt receptors on neural stem cell, and activates non-canonical Wnt/PCP/JNK or Wnt/Ca2+ signaling. Activation of noncanonical *Wnt* pathway results in activation of synaptogenesis and axon guidance. (Data collection and analysis by K. Ingraham and M. Woodbury, Figure by H. Yeh and M. Woobury.

Here, we show that mouse microglia express Wnt using microarray analysis across all conditions (**Figure 5.3a**). *Wnt5a* is the most abundantly expressed *Wnt* gene in microglia *in vitro,* followed by Wnt7a. We also show that WNT5a was most abundantly expressed in microglia than astrocytes and oligodendrocytes (**Figure 5.3b-c**). Previously,

Halleskog et al. showed astrocytes to express higher levels of WNT5a (Halleskog et al. 2011). This may be due to the age of cells since microglial Wnt5a expression peaks during the embryonic stage and reduces with aging (Bennett et al. 2016). Expression of WNT5a peaks at the embryonic and early neonatal stage in mouse cerebellum (Dionisio-Santos, Olschowka, and O'Banion 2019). On the other hand, human microglia had the highest expression of WNT5a, suggesting microglia may play a more significant role in Wnt5a signaling in the human brain (**Figure 5.3d-f**) (Bennet et al. 2016; Zhang et al. 2014; Zhang et al. 2016). Among the glial cells, immature microglia are the most dominant glial cell to express Wnt5a. Therefore, we hypothesized that microglial Wnt5a is critical in mediating neurodevelopment.

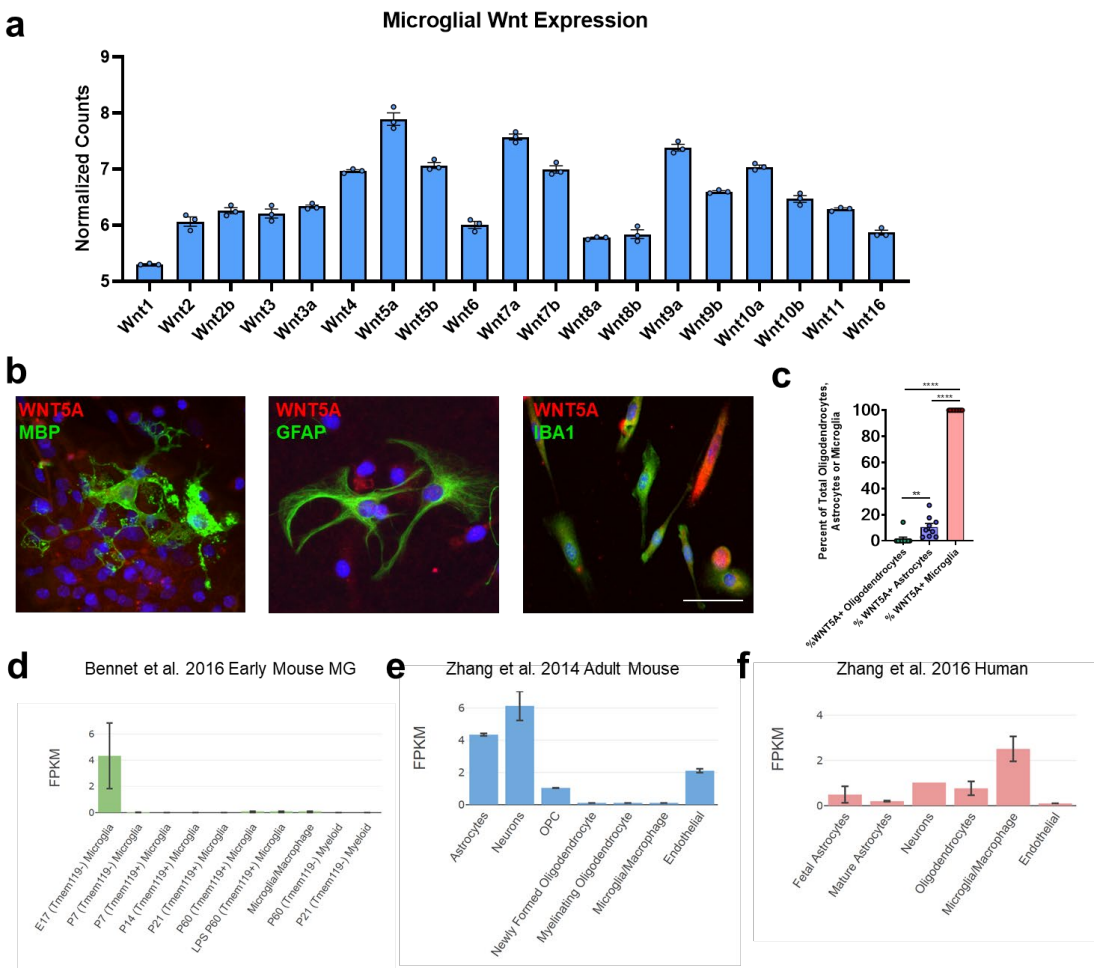


Figure 5.3. Wnt5a is highly expressed in microglia compared to other Wnts and other neural cell types.

a) Microarray gene analysis of *Wnt* expression in microglia. **b)** Representative images of Wnt5a expression in different glial cell types. Wnt5a is shown in red, myelin basic protein (oligodendrocytes), GFAP (astrocytes) or IBA1 (microglia) shown in red. DAPI shown in blue. **c)** Quantification of %Wnt5a+ cells shown in (b). Scale bar denotes 50um. *** denotes $p<0.001$ vs. astrocytes, ### denotes $p<0.001$ vs. oligodendrocytes by One way ANOVA with Tukey post-hoc. **d)** Bennet et al. early immature microglia expression of Wnt5a. **e)** Zhang et al. adult microglial expression of Wnt5a. **f)** Zhang et al. human microglia expression of Wnt5a. Bars show mean +/- SEM. Data collection by K. Ingraham and M. Woodbury. Figure by H. Yeh and M. Woobury.

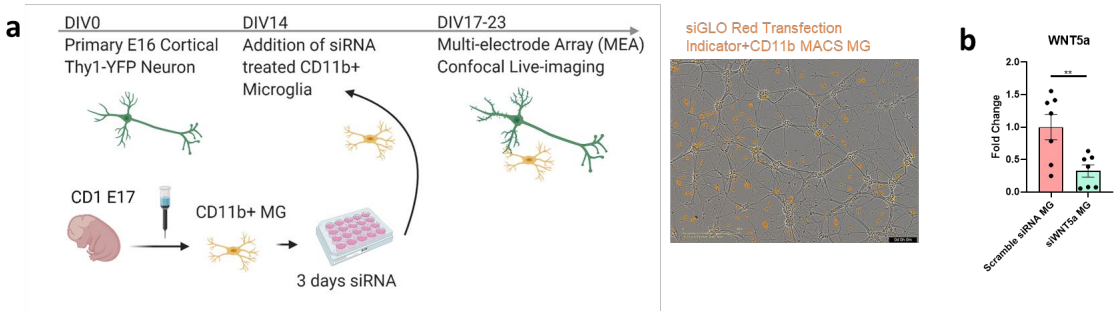


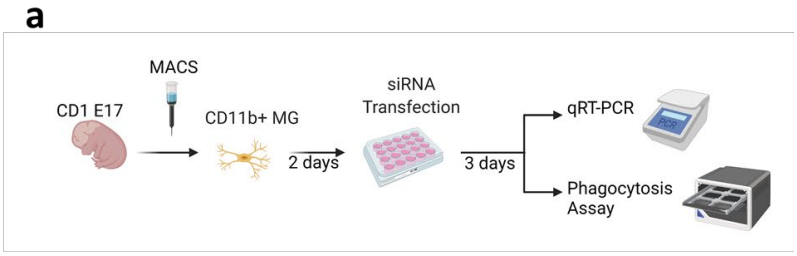
Figure 5.4. Silencing microglia WNT5a by siRNA and co-culturing with primary neurons

a) Experimental design for silencing WNT5a in CD1 cortical E17.5 microglia and subsequent co-culture with primary cortical neurons from E16 Thy1-YFP mice. Silencing of Wnt5a achieved by Accel siRNA transfection at 1uM in transfection media. **b)** qRT-PCR confirmation of silencing Wnt5a in CD1 primary microglia at DIV8. (N=7 replicates, three independent experiments). Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Then, we confirmed microglia WNT5a could be reduced through siRNA transfection (**Figure 5.4a-b, Figure 5.5a**). mRNA was purified from microglia at DIV7, 4 days following complete removal of siRNA-containing transfection media. qRT-PCR was performed to confirm the knockdown of WNT5a and found 67% reduction of *Wnt5a* mRNA expression. siRNA silencing of WNT5a led to downregulated phagocytosis gene *C1qa* expression (**Figure 5.5b**), consistent with previous findings in macrophages (Maiti, Naskar, and Sen 2012). But no changes in microglial genes such as homeostatic gene *P2ry12* or disease-associated gene *Clec7a* (**Figure 5.5b**). Single-cell RNA-seq studies reveal that fetal microglia are highly phagocytotic and share enrichment of DAM/MGnD microglia genes, suggesting common microglia pathways activated in the embryonic stage and neurodegenerative disease (Masuda et al. 2019; Li et al. 2019). With increased age, microglia acquire more homeostatic phenotype with enrichment of microglia

sensome genes for immune sensing and surveying the brain parenchyma (Kracht et al. 2020). These qPCR results show that WNT5a may have minimal impact on microglia maturation but rather affect phagocytosis function.

Wnt5a is also known to activate microglia by increasing inflammation-related gene expression, including IL-1b, IL-6, and TNFa (Halleskog et al. 2011). Wnt5a is upregulated in response to mycobacterial pathogen, LPS and interferon-gamma treatment, suggesting WNT5a can regulate inflammatory response to pathogens (Blumenthal et al. 2006; Pereira et al. 2008). Maiti et al. found that WNT5a suppression inhibited phagocytosis in macrophage cell lines that was accompanied by reduced TNFa and IL-6 production and increased IL-10 secretion, suggesting Wnt5a may be crucial in maintaining microglial immune function (Maiti, Naskar, and Sen 2012). Here we show that silencing Wnt5a in microglia only enhanced *Il1b*, suggesting reduced Wnt5a expression had a minimal impact on the immune response (**Figure 5.5c**). This discrepancy may be due to the nature of macrophage cell lines that have enhanced phagocytosis and activation status . Changes in *C1qa* and *Il1b* by silencing microglial WNT5a suggest a possible alteration in phagocytosis function. However, phagocytosis assay using *E. Coli* pHrodo bioparticles show that microglial Wn5a-deficiency had no significant impact on phagocytosis function *in vitro* (**Figure 5.5d**). Despite changes observed by qRT-PCR, microglial *Wnt5a* may not be the primary mediator of phagocytosis function.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Transfection
3 days
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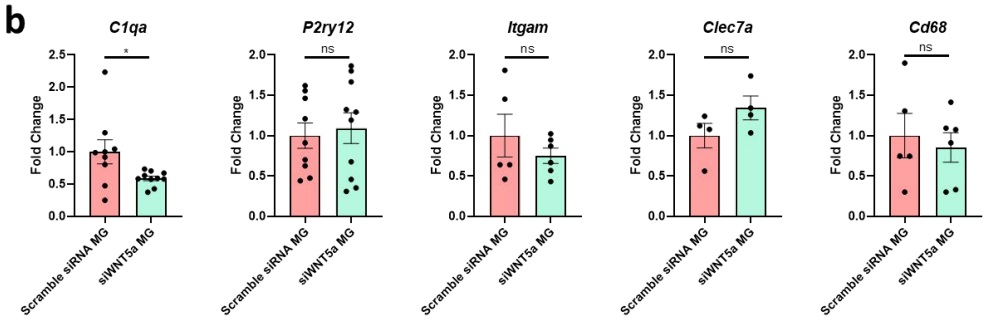
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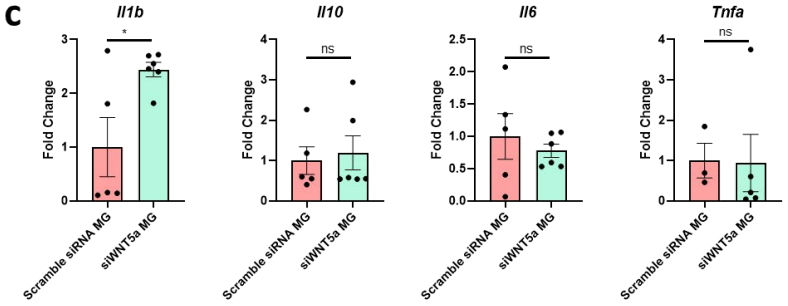
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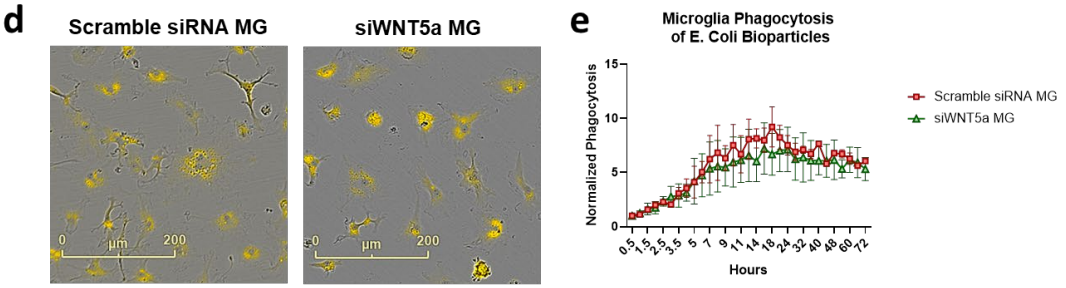
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Figure 5.5 Silencing of microglial WNT5a had minimal impact on microglia markers

a) Experimental scheme of primary microglia culture and siRNA transfection treatment to silence WNT5a. **b)** qPCR quantification of silencing WNT5a on microglial markers *C1qa*, *P2ry12*, *Itgam*, *Clec7a*, *Cd68*. Silencing microglia WNT5a reduced phagocytosis marker C1qa. **c)** Microglial cytokine expression largely unchanged by silencing of WNT5a except pro-inflammatory cytokine IL1b. Unpaired t-test for statistical analysis. **d)** Representative images of E17.5 primary microglia pHrodo E coli bioparticles at 72 hours. **e)** IncuCyte quantification of microglia phagocytosis of scramble siRNA or siWNT5a treatment. Normalized phagocytosis quantified by number of pHrodo+ microglia relative to number of microglia cells per well (n=4 replicates, 2 independent batches). Two-way ANOVA analysis, Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

The silencing of WNT5a in microglia can affect intercellular communication, thereby changing neural circuitry. We characterized the effect of WNT5a in co-cultures of microglia and neurons (**Figure 5.4a**). At D17, the addition of microglia increased both dendritic length and the number of Sholl intersections, suggesting that microglia can promote dendrite growth and complexity (**Figure 5.6a-b**). Silencing of microglial WNT5a has no significant differences compared to the control scamble siRNA-treated microglia, suggesting a minimal impact of microglial Wnt5a on neurite development. Wnt5a decreases with age and is associated with loss of dendritic arbors and spines in CA1 with impaired cognitive behavior (Chen et al. 2017). Exogenous WNT5a stimulation in aged mice prevented age-related dendritic regression, suggesting a possible role in preventing age-related neurodegeneration (Chen et al. 2017). Another study showed that WNT5a acts as a repulsive signal and reduces neurite length of dopaminergic neurons, suggesting WNT5a action on neurons is likely cell-type and region-dependent (Blakely et al. 2011). Notably, Wnt5a administration to more immature

cortical neurons promotes axonal but not dendritic outgrowth, suggesting the timing of WNT5a activation can be critical in neural differentiation and neurite development (Horigane et al. 2016). Here, we report that microglia can provide a non-neuronal source of WNT5a in regulating neuron morphology.

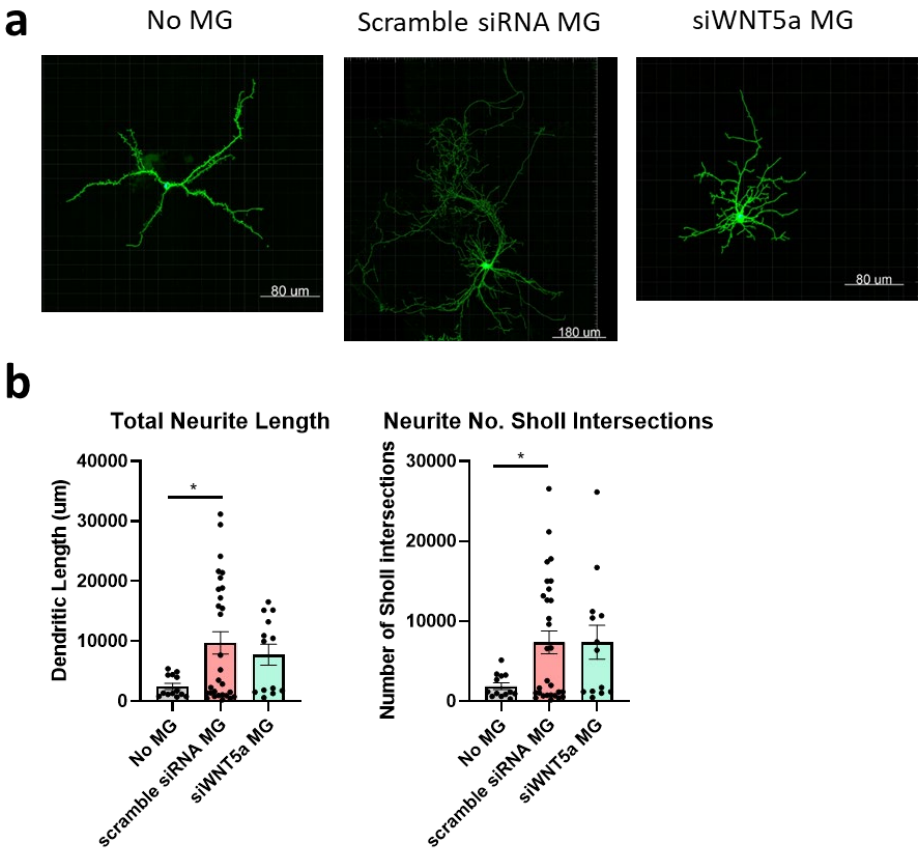


Figure 5.6 Microglial Wnt5a enhanced neurite development at DIV17

a), Representative images of Thy1-YFP neurons at DIV17 with or without microglia co-culture**. b),** Quantification of total neurite length or neurite number of Sholl intersections by IMARIS analysis of Thy1-YFP+ at DIV17. N=13/29/13 as No MG/scramble siRNA MG/siWNT5a MG respectively, from 3 independent experiments. Statistical analysis using Kruskal-Wallis and Dunn's multiple comparison test. *P<0.05. *p<0.05. Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Live imaging was performed to examine spine density and turnover rate at D22-23 to examine if microglial WNT5a affected spine growth and maintenance. We utilized Thy1-YFP+ neurons to visualize dendritic spines in vitro since studies have shown that Thy1-expressing layer V neurons require microglial-secreted factors for survival and proper differentiation (Ueno et al. 2013). Thy1-YFP+ dendritic spines became visible and more mature, forming mushroom-like spines at DIV22-23 for imaging spine density and spine turnover. The spine density of Thy1-YFP^{+} neurons was increased upon microglial addition, which was reversed upon siRNA silencing of WNT5a at D22-23 (**Figure 5.7a-c**). Previous *in vivo* studies confirmed microglia depletion elevated spine turnover, and the generation of pre-synaptic filopodia are microglia-dependent processes, suggesting a critical role of microglia in spine turnover (Cangalaya et al. 2020). Thus, we determined if silencing microglia WNT5a can affect the spine turnover rate *in vitro*. The appearance and elimination of spines were quantified over 15 minutes of imaging to assess spine stability since immature spines have a high turnover rate, which is defined by the sum of the protrusions lost and gained divided by twice the total protrusion number as previously defined (**Figure 5.7b-c**)(Cruz-Martín, Crespo, and Portera-Cailliau 2010). Interestingly, silencing of WNT5a in microglia increased the turnover rate of spines compared to scramble siRNA-treated microglia, suggesting microglial Wnt5a mediates dendritic spine stability and maintenance (**Figure 5.7c**). Overall, this indicates that microglia co-culture enhanced spine density and increases the stability of spines as indicated by lower spine turnover rate compared to no microglia and silencing of microglial Wnt5a.

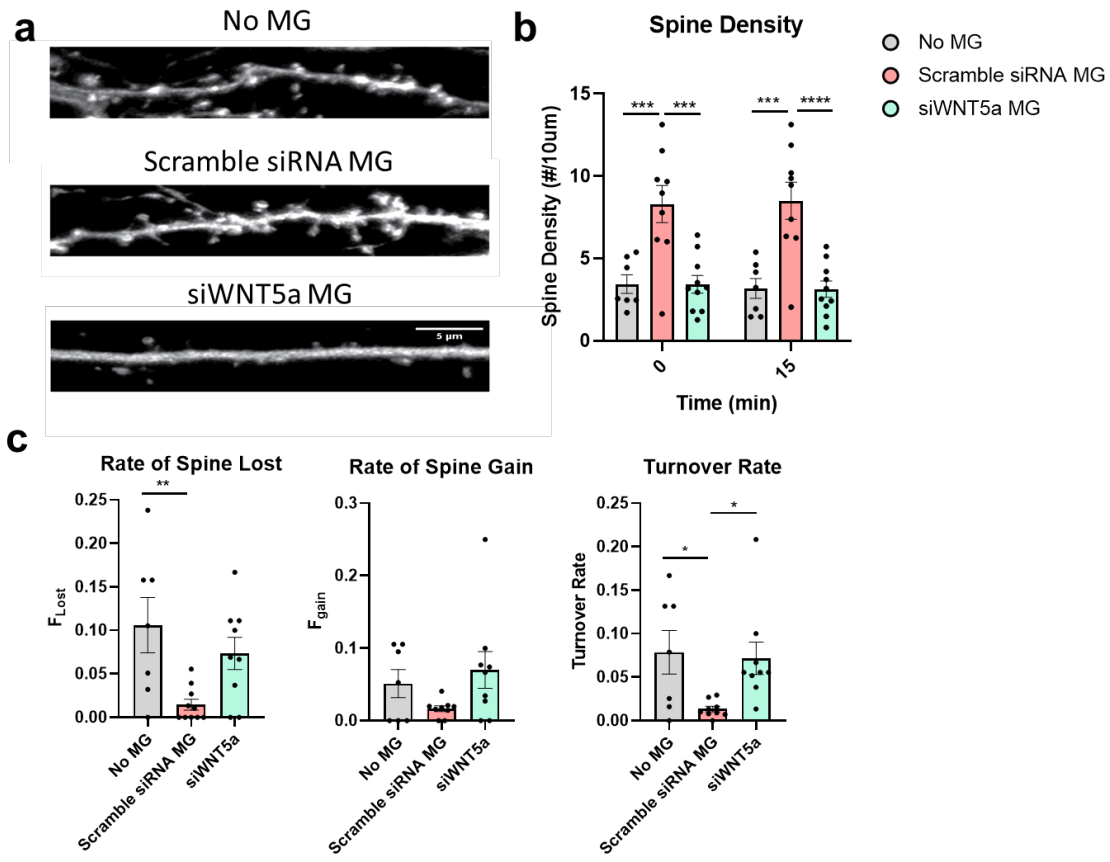


Figure 5.7 Microglia increases spine density and stabilizes spine maintenance at DIV22-23

a) Representative images of Thy1-YFP neurons co-cultured with microglia treated with scramble siRNA or siWNT5a. scar bar= 5um. **b)** Live imaging of dendritic spine density over 15 min interval. Analysis performed by Two-way ANOVA and Sidak's multiple comparison to determine time and microglial treatment interaction. **c)** Microglia presence reduced spine turnover rate, and silencing of microglia WNT5a increased spine turnover rate. F_{gain} and F_{lost} were respectively defined as the ratio of spines that appeared and disappeared between two successive frames over the 15 min imaging period, relative to the total spine number. Spine turnover rate is the sum of the protrusions lost and gained divided by twice the total spine number over 15 min interval to measure stability of Thy1-YFP dendritic spines. For c, statistical analysis using Kruskal-Wallis and Dunn's multiple comparison test. *P<0.05. Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Previous studies show exogenous WNT5a treatment significantly increased PSD-95 clustering and induced mature dendritic protrusions via calcium-dependent intracellular pathways (Farías et al. 2009; Varela-Nallar et al. 2010). To determine pre- and post-synaptic changes due to microglia, we performed immunocytochemistry for pre-synaptic VGLUT2 and post-synaptic PSD95 (**Figure 5.8a**). We found no differences in the total number of VGLUT2 puncta by microglia addition at DIV23 (**Figure 5.8c**). The addition of microglia significantly increased the PSD95 density and synapse co-localization with VGLUT2 *in vitro* (**Figure 5.8d**). Microglial Wnt5a-deficiency led to a trend in reduced synapse colocalization of PSD95 and VGLUT2, which corresponds to reduced dendritic spine density at DIV22-23. Together, these results suggest that microglia can secrete WNT5a and regulate neuronal excitability via a postsynaptic mechanism in Thy1-YFP^{+} neurons.

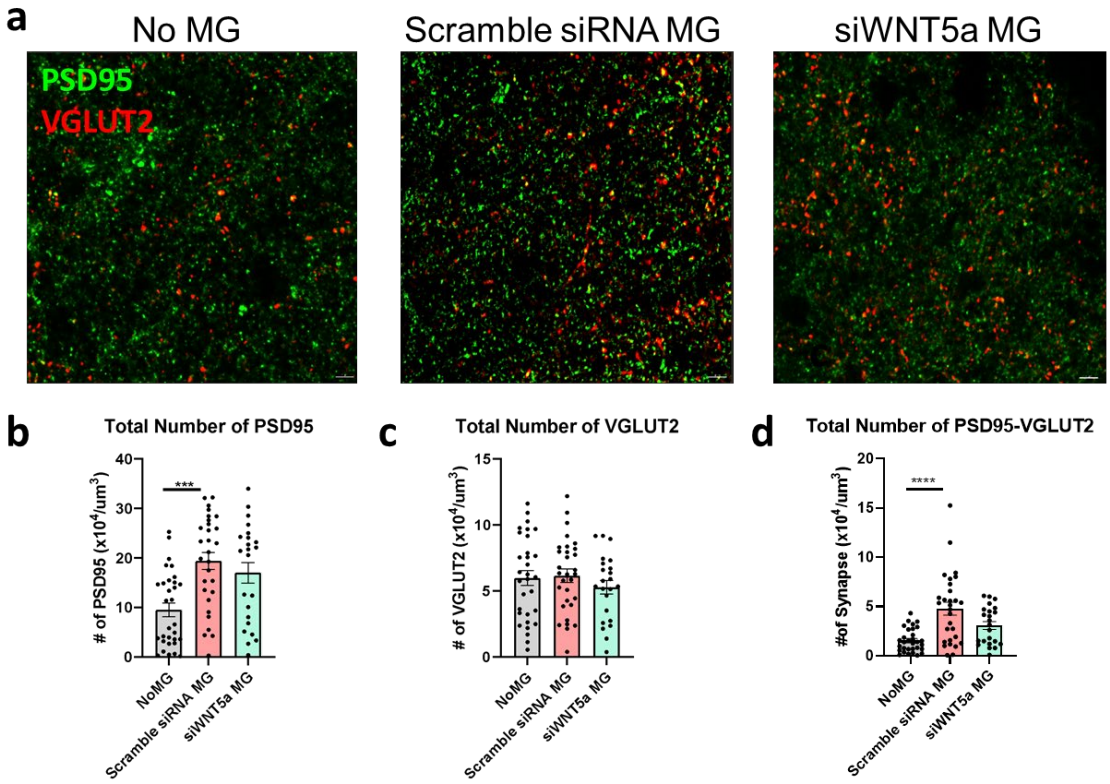
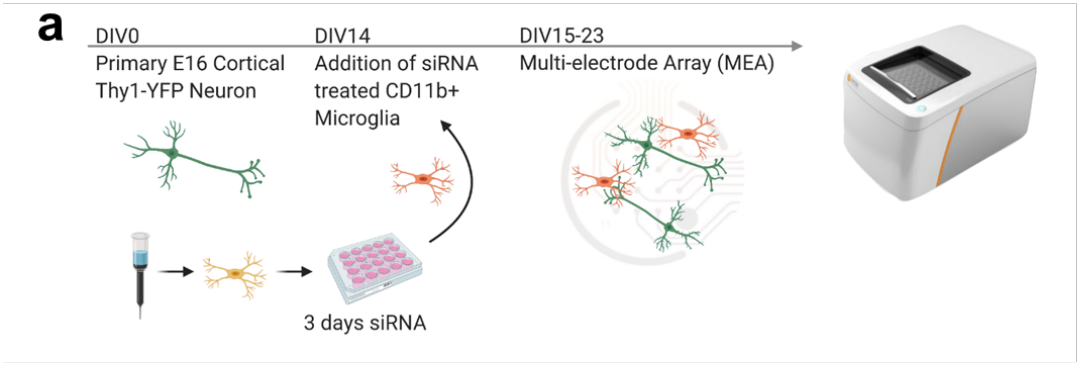


Figure 5.8 DIV22-23 live imaging of Thy1-YFP$^+$ neurons show altered dendritic spine density and turnover rate.

a) Immunocytochemistry representative images of primary neurons co-cultured with microglia for pre-synaptic VGLUT2 (Red) and post-synaptic PSD95 (Green) markers. **b)** Total number of PSD95 normalized by volume increased by addition of microglia. **c)** Total number of VGLUT2 normalized by total volume showed no significant differences between no microglia compared to addition of microglia with or without siRNA WNT5a. **d)** Total number of co-localization of PSD95 and VLGUT2 synapse density normalized by volume was enhanced by microglia co-culture, and reduced by silencing microglia WNT5a. Statistical analysis using Kruskal-Wallis and Dunn's multiple comparison test. *P<0.05. (n=31, 29, 23 from 3 independent batches of neuron and microglia culture). Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Lastly, we recorded neuronal spikes and burst using the multi-electrode array since DIV15, one day after the addition of microglia to primary neurons, to test if

microglial WNT5a can affect neuronal activity (**Figure 5.9a**). Despite a significant effect of microglia treatment on dendrite growth, neuronal spikes were not significantly altered at DIV17 (**Figure 5.9b**). This could suggest that microglia could potentially alter intrinsic excitability by increasing dendritic branching length and complexity but not firing rate at this time point. At DIV22, we observed that the addition of microglia enhanced neuronal action potential firing rate and silencing of microglial WNT5a had a diminished effect on firing rate (**Figure 5.9b**). WNT5a increases the efficacy of glutamatergic synaptic transmission via increased intracellular calcium and NMDARs trafficking (Varela-Nallar et al. 2010; McQuate, Latorre-Esteves, and Barria 2017). Thus, microglia Wnt5a may promote neuronal firing by upregulation of postsynaptic PSD95 or inducing Ca2+ influx. There was no significant effect of microglia on neuronal burst and no effect on neuronal synchrony, suggesting microglia acts differently from astrocytes, which enhances neuronal synchrony (Fellin et al. 2004). However, glutamate signaling mechanisms are complex and influenced by many factors. Examination of structural components of glutamatergic synapses, including AMPA/NMDA receptors, can determine the microglial effect on intrinsic excitability. Whole-cell patch-clamp can be performed *in vitro* to record membrane resistance, action potential properties, and excitatory postsynaptic currents in future studies.



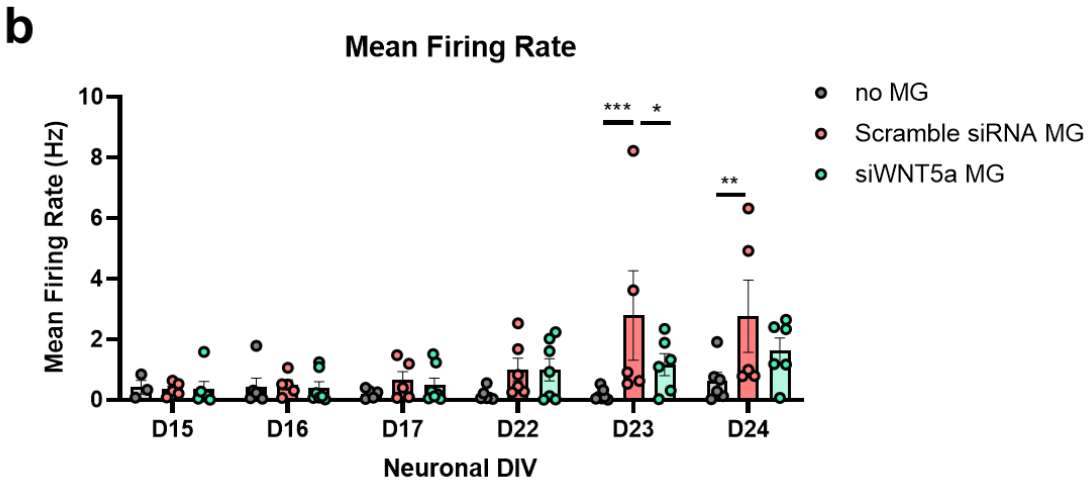


Figure 5.9 Microglia enhanced neuronal firing rate at DIV23-4, but diminished by silencing of microglia WNT5a

a) The multi-electrode assay examining silencing WNT5a in microglia co-cultured with primary cortical neurons isolated from Thy1-YFP E16 mice at DIV16-23. **b)** Mean firing rate was measured over DIV16-23, with the most significant increase at DIV23-24. Two-way ANOVA analysis performed to determine time and treatment interaction effect. (n=3-6 replicates, 3 independent batches of neuron and microglia culture, one outlier removed at DIV15 in siWNT5a, one outlier removed each at DIV16, DIV22, DIV23 in no MG group) **p<0.01, ***p<0.005. Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

5.4 Discussion

The Wnt pathway is implicated in both neurodevelopment and neurodegeneration disorder. Activation of canonical Wnt signaling or suppressing noncanonical Wnt pathways can lead to neuroprotective effects in a transgenic mouse model of Alzheimer's disease and reverse hippocampal-dependent deficits in a mouse model of Fragile-X syndrome (Li et al. 2011; Chacón, Varela-Nallar, and Inestrosa 2008; Guo et al. 2012; Oliva, Vargas, and Inestrosa 2013). Noncanonical Wnt5a regulates neuronal differentiation and specialization, proper axon guidance, and glial progenitor cell proliferation in the cerebellum (Keeble et al. 2006; Blakely et al. 2011; Subashini et al. 2017). Knockout WNT5a mice show impaired developmental processes, including the inability to extend the anterior-posterior and proximal-distal axes of the embryo (Yamaguchi et al. 1999; Kikuchi et al. 2012). Some have shown enhanced axon defects of dopaminergic neurons in the dorsal-lateral striatum (Bian et al. 2015; Varela-Nallar et al. 2010). In contrast, Chen et al. reported embryonic deletion of WNT5a in neurons did not result in any structural abnormalities in CA1 pyramidal neurons during development suggesting neuronal WNT5a mediates synaptic plasticity rather than maturation (C.-M. Chen et al. 2017). Thus, non-neuronal effects of WNT5a may play a more critical role in early development.

Microglial contact leads to filopodia induction (Weinhard et al. 2018; Miyamoto et al. 2016). We previously showed enhanced microglia-neuron contact in MIA offspring, in which WNT5a expression is increased in MIA microglia (Ikezu et al. 2020). In this study, we show that the microglial-derived secretome molecule WNT5a can mediate

early neurodevelopment. In particular, we found that the microglial secretome can promote neural differentiation without direct microglia-neuron contact. Based on pathway analysis, microglia can mediate neuronal development via Wnt pathways. Thus, we hypothesize that WNT5a is secreted by immature microglia and binds to ROR1/2 receptors to activate WNT/Ca+ and WNT/JNK pathways for neuronal differentiation. We found Wnt5a to be the top-expressed Wnt in embryonic microglia and that it can be silenced by siRNA transfection with minimal effect on microglia phenotype and immune response. Wnt signaling may include autocrine pathways, which suggest that microglial Wnt5a may also mediate microglial profile by binding to its ROR/FZD receptors and activating non-canonical Wnt pathways (Luga et al. 2012; Ryu et al. 2013). Silencing of WNT5a in microglia can alter the autocrine Wnt signaling pathway that can activate microglia, as indicated by increased IL-1b expression. This can be further assessed by depleting Wnt5a in microglia media by anti-Wnt5a and determining cytokine secretion and inflammation markers in siWNT5a or control microglia. Microglia co-culture enhanced neurite development and dendritic spine density in Thy1-YFP neurons. Microglial-derived Wnt5a enhanced spine maturity and promoted synapse colocalization of PSD95 and VLGUT2 via a postsynaptic mechanism, leading to increased neuronal activity. Further validation using *in vivo* system is necessary to examine the physiological relevance of microglial Wnt5a on neurite development and synapse maintenance.

Other Wnts are critical to early neurodevelopment. Wnt3a, Wnt2b, and Wnt8b are also involved in the regulation of patterning and neurogenesis in the dorsal cortex (Lie et al. 2005; Munji et al. 2011). Canonical Wnt3a and Wnt7a mediate recycling and

exocytosis of synaptic vesicles in mature hippocampal neurons and enhance synaptic transmission in adult hippocampal brain slices (Cerpa et al. 2010). WNT3a also suppresses dendrite maturation of subventricular zone-derived neurons *in vitro* (Pino, Choe, and Pleasure 2011). Noncanonical Wnt7b has been associated with dendritic complexity by activating Rac and JNK pathways (Rosso et al. 2005). Further investigation on how other Wnt ligands secreted by microglia affect neural development is required to understand how microglial Wnt can support proper brain function.

CHAPTER SIX

Developing *in vitro* inducible immortalization of a microglia cell line for high throughput studies

6.1 Introduction

In vitro models of microglia have been developed over recent decades, including primary dissociated cell cultures, microglia cell likes, and stem cell-derived microglial-like cells. Microglia culture models have been developed to recapitulate the characteristics and developmental processes of microglia *in vivo*, but present many limitations. Microglia usually lose their distinct expression patterns with increasing time cultured *in vitro* (Gosselin et al. 2017). Cultured microglia become more activated and show amoeboid morphology, increased cell proliferation, and heightened phagocytic activity that is also dependent on culture media composition (Kettenmann et al. 2011). Many of these artifacts can be reversed by engraftment into microglia-deficient brains, suggesting that CNS-specific cues are required to sustain microglia specification and promote homeostasis phenotype (Bennett et al. 2016; Bohlen et al. 2017).

Microglia cell lines of mouse, rat, and human origin are usually immortalized by viral transduction with various oncogenes. The currently available microglial cell lines have advantages, including ease of maintenance and their unrestricted proliferation capacity. The most commonly used microglial cell line BV2 was generated by transduction of neonatal primary microglia from v-raf/v-myc carrying J2 retroviruses and express macrophage markers including MAC1 and MAC2 (Timmerman, Burm, and Bajramovic 2018). BV2 cells are well-characterized, including their phagocytosis

capabilities and expression of inflammatory markers in response to LPS stimulation. However, these cells are also prone to dedifferentiation and alteration of microglial phenotype. Due to the constant expression of oncogenic genes, BV2 cells are often regarded as activated microglia-like cells with rapid proliferation properties. The rapid proliferation rate of BV2 cells makes it more challenging to maintain and assess certain microglia functions reflecting a more homeostatic state.

There can also be a significant batch effect for primary microglia culture due to the background of mice or the isolation purity of microglia. Here, we propose a method to immortalize murine microglia in an inducible manner that enables rapid expansion of single-cell colonies with minimizing activation status associated with the rapid growth of the microglial population. Immortalization enables high-throughput testing for drug screening or for many gene targets simultaneously without confounding factors such as purity of isolation and batch effect.

6.2 Methods

Molecular cloning and lentiviral production

A mutant form of HRAS G12V containing P2A sequence was synthesized by GenScript (NJ, USA) based on the published sequence of HRAS G12V (Kendall et al. 2005). PU57 plasmid containing HRAS G12V was subsequently cloned into the lentiviral vector with a tetracycline response element with CMYC T58A (Maherali et al. 2008) Addgene Plasmid #19775) using BsaBI and Xba1 restriction enzymes. The lentiviral TET-O-CMYC T58A-P2A-HRAS G12V and rTTA plasmids (Maherali et al. 2008;

Addgene plasmid #19780) were amplified using Sure2 E. coli cells to produce stable plasmids. The cloned plasmid was sequenced for confirmation of correct insertion. Lentivirus stocks were generated using HEK 293FT cell lines co-transfected with lentiviral TET-O-CMYC T58A-P2A-HRAS G12V, and three packaging plasmids (pLP1, pLP2, and Vsvg) using Lipofectamine 2000 according to manufacturer's protocol and previous study. Lentiviral particles were harvested at 2-3 days post-transfection following complete media change 24 hours following transfection. Lentiviral particles were concentrated using ultracentrifugation and stored at -80C.

Microglia culture and lentiviral transduction

Primary microglia were isolated from CD1 E17.5-18.5 embryos using Embryonic Neural Dissociation kit microbeads (130-093-231, Miltenyi Biotec) and CD11B microbeads (130-093-634, Miltenyi Biotec) according to the manufacturer's protocol. Primary microglia cells were plated in DMEM/10% FBS media supplemented with 20ng/ml M-CSF (416-ML-010/CF, RD) at 100,000 cells per well in a 24-well plate. Media was refreshed by changing half of the media every three to four days. At DIV4, half of the media was changed and supplemented with 1ug/ml polybrene. TET-O-CMYC T58A-HRAS G12V and rTTA lentiviral particles were added to media at days in vitro (DIV) 4 at 1ug/ml. Complete media was changed the following day and was replaced with DMEM/10%FBS supplemented with 20ng/ml MCSF and 2.5 ug/ml doxycycline to induce overexpression of CMYC T58A and HRAS G12V. Small molecules SB431542 (SB, 3014193, PeproTech), GSK3β inhibitor CHIR99021(CHIR, 13122, Cayman

Chemical Company), and valproic acid (VPA, 13033, Cayman Chemical Company) were added to media after dissolving in PBS.

FACS and flow cytometry

Media was aspirated and washed with PBS. Microglial cells were then dissociated from the plate by cold PBS and pipetting up and down. Cells were resuspended in 0.5%BSA in PBS for staining. Cells were stained with the live/dead Blue kit (L34961, ThermoFisher) to remove dead cells. Cells were stained with CD11B-PeCy7 (25-0112-82, Life Technologies), CD45-BV421 (30-F11, BD Biosciences), CX3CR1-APC (341609, Biolegend) as previously described (Ikezu et al. 2020). CD11b+ sorted cells were then plated for continued culture.

Immunocytochemistry

For immunocytochemistry, cells were plated on poly-D-lysine coated glass coverslips. Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Following repeated PBS wash, cells were permeabilized with 0.1% Triton-X in PBS. Then, cells were treated with blocking buffer (5% Normal Donkey Serum/NDS in PBS) for 1.5 hours and incubated with staining buffer (5% NDS, 0.1% Triton-X, 0.2%BSA) overnight at 4C with primary antibodies 1:1000 dilution. The following primary antibodies were used: TREM2 (AF1729, Biorad), rabbit-IBA1 (019-19741, WAKO). Cells on coverslips were washed with PBS and incubated with secondary antibodies for 1 hour at room temperature. Nuclear staining was achieved using Hoechst 33342 1:20,000 in PBS dilution. Coverslips were washed and mounted using Flouromount (Invitrogen).

Live imaging: phagocytosis and scratch-wound assay

For phagocytosis assay, cells were dissociated with 30% Accutase and placed in 48-well plates. A complete media change was performed the next day and growth factors or doxycycline were added. After 3 days of incubation with or without treatments, media was changed and pHrodo *E. Coli* bioparticles (4615, Sartorius) at 10ug/ml were added immediately before imaging. Both phase and orange channel were captured to assess microglial confluence and fluorescence of internalized bioparticles. Images were captured by 20x IncuCyte (Sartorius) every 15 minutes for the first 3 hours followed by 1-h intervals.

For scratch wound assay, cells were passaged and plated in 96-well plates. Complete media change was performed the next day and 20ng/ml M-CSF or 2ug/ml doxycycline were added. After 3 days of incubation with or without treatments, media was changed After cells reached confluence, a scratch wound was created using a P20 pipette tip. Debris was washed by complete media change with or without 20ng/ml M-CSF. Images were captured by 20x IncuCyte (Sartorius) every 30 minutes.

LPS stimulation and ELISA

Following passaging and placing cells into a 12-well plate, the media was completely changed to DMEM/10% FBS with or without 20ng/ml M-CSF. Three days following media change, microglia were stimulated by 1ug/ml [check concentration] LPS. Media was collected at 3 and 24 h following LPS stimulation. ELISA was performed according to the manufacturer's protocol. Following ELISA kits were used to detect IL-6 (431304, Biolegend), IL-1b (ab197742, Abcam), TNFa (430901, Biolegend),

and CCR2/MCP-1 (446207, Biolegend). Expression levels were normalized based on total cell lysate protein using BCA (23225, ThermoFisher Scientific).

6.3 Results

First, CD11b+ microglial cells were isolated from neonatal CD1 E18.5 embryos using MACS for primary cell culture. Various conditions and overexpression of genes have been tested to induce immortalization of mouse microglia (**Table 6.1**). Small molecules consisted of TGF-β inhibitor SB431542 (SB), GSK3β inhibitor CHIR99021(CHIR), and valproic acid (VPA), which have no significant effect on long-term cell expansion and renewal. Growth factors thrombopoietin (TPO) and stem cell factor (SCF) did not enhance self-renewal in primary mouse microglia, suggesting that external molecules or growth factors are insufficient to induce continuous self-renewal. The most efficient inducible immortalization strategy combines CMYC T58A and HRAS G12V oncogenic induction using a lentiviral TET-O system with rTTA in mouse microglial cells. The mutant form T58A of CMYC enabled enhanced self-renewal capacity and increased proliferation with more clonogenic potential without tumorigenicity for neural stem cells (De Filippis et al. 2008). We found doxycycline induction stimulated significant cell proliferation in TET-O-HRAS-CMYC treatment compared to control following two weeks of treatment (**Figure 6.1a-c**). Immunocytochemistry revealed most isolated murine cells express microglial marker IBA1 following three weeks of incubation (**Figure 6.1b**). Based on live imaging over 7 days, the transduced microglial cells doubled in cell density within 4 days upon

doxycycline treatment for oncogenic expression (**Figure 6.1c).** The results show successful transduction of primary IBA1+ microglia cells and induction of microglial proliferation by doxycycline treatment.

Factors	**Molecules**	**Concentration**	**Outcome**
TERT/MYC	SB, CHIR, VPA ↓FBS	10%FBS vs 2% FBS	Reduction of FBS without replacement not healthy
TERT	SB, TPO+ SCF+ PVA	10ug/ml, 100ng/ml, 10ng/ml, 10ng/ml-100mg/ml	Not proliferative
MYC			Not proliferative
TERT/MYC	SB, CHIR, VPA ↓FBS	10%FBS vs 2% FBS	Reduction of FBS without replacement not healthy
CMYC T58A / HRAS G12V	MCSF	25ng/ml MCSF in DMEM/10% FBS	FACS of CD11b+CD45+ mouse microglia-> expansion-> able to select single cell colony and proliferate -> able to freeze/thaw for passage -> ongoing

Table 6.1. Experimental conditions tested for immortalization of microglia of human and mouse

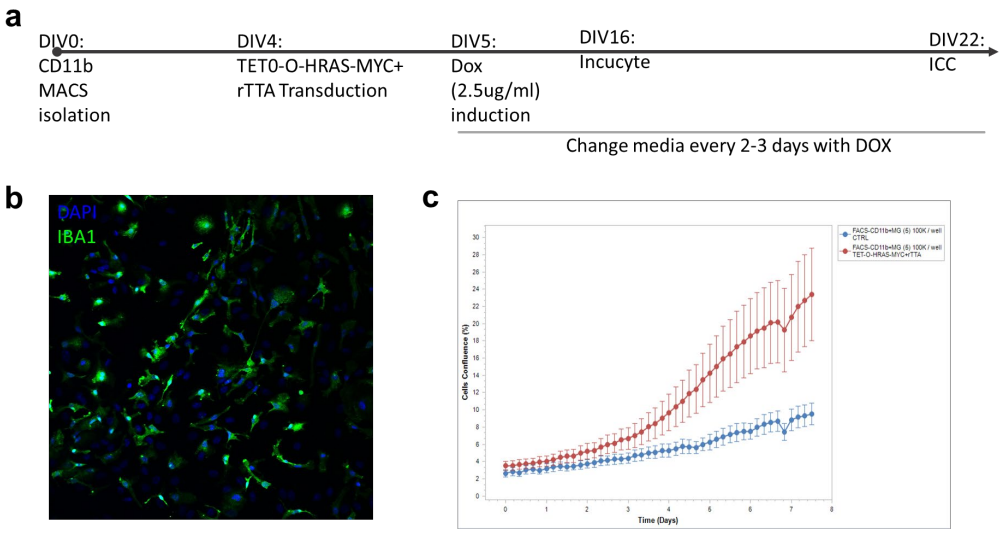


Figure 6.1. Experimental scheme for microglial immortalization.

a) Timeline of transduction of primary CD1 E17.5 microglial cells at DIV4 and doxycycline induction of CMYC T58A and HRAS G12V at DIV5. **b)** Immunocytochemistry of IBA1+ (Green) non-transduced control microglia showing high purity microglia culture. **c)** Cell confluence by live IncuCyte cell imaging of microglia cells transduced with TET-O- CMYC T58A-HRAS G12V or nontransduced microglia at DIV16-23. Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Following DOX induction of CMYC T58A and HRAS G12V overexpression, primary microglial cells were sorted for CD11b+LY6C- to obtain a higher purity of microglial cells (**Figure 6.2a-b**). In transduced microglial cells with CMYC T58A and HRAS G12V, the percentage of CD11B+LY6C- was decreased to around 3.9% of the total live cells compared to 30.1% in the control non-transduced microglial cells, indicating doxycycline-induced overexpression of oncogenes reduced the relative proportion of CD11b+ microglial cells (**Figure 6.3a**). Doxycycline-induced reduction of CD11b+ expression can be caused by inducing a proliferative and immature microglia state or expansion of non-microglial cells. Hence, FACS was required to remove

contaminating cells following MACS CD11b+ microglia isolation and doxycycline induction. Further confirmation using qRT-PCR revealed that oncogenes CMYC and HRAS were significantly upregulated following DOX induction. Microglial genes *C1qa* and *P2ry12* expression were significantly reduced, suggesting overexpression of oncogenes led to the loss of microglial identity during the proliferation phase (**Figure 6.2b**). Both oncogenes *CMYC* and *HRAS* were successfully induced by doxycycline induction by at least 1000x fold. Single-cell colony expansion was performed to obtain homogenous microglial populations and determine if CMYC T58A-HRAS G12V overexpression can enable single-cell expansion. We successfully obtained 4 single-cell colonies (clone 2E11, 2D4, 1F3, and 1H9 mMG) from 2 96-well plates. The following microglia characterization focused on clone 2E11mMG based on its robustness in self-renewal.

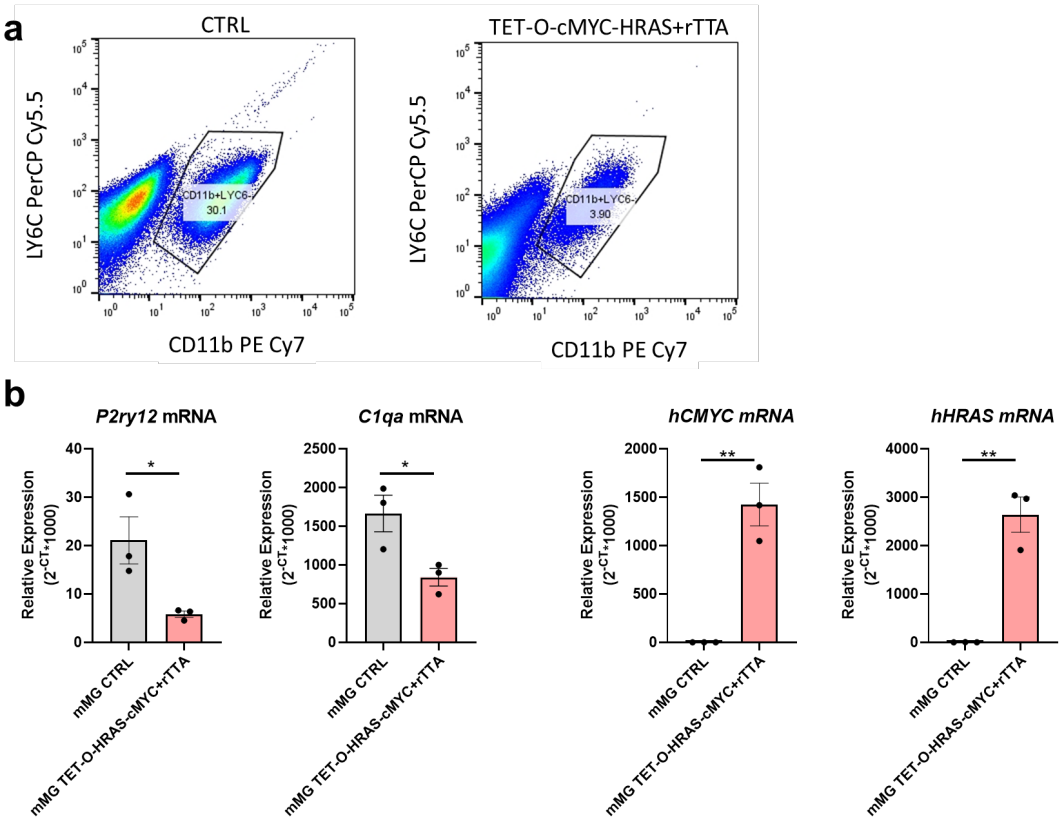


Figure 6.2. FACS CD11b and qPCR of microglia and CMYC and HRAS genes.

a) FACS gating of CD11B+LY6C- cells for microglial single cell colony expansion
b) Relative expression of microglial genes *C1qa* and *P2ry12*, and oncogenes *CMYC*, *HRAS* normalized to *Gadph* using qRT-PCR of CD11b+LY6C- microglia cells comparing control versus transduced TET-O-CMYC T58A-HRAS G12V with doxycycline microglia cells. Statistical analysis by unpaired t-test. N=3 replicates Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

We hypothesized that doxycycline-induced expression of oncogenes could enable rapid expansion of 2E11 microglia cells, but the withdrawal of doxycycline allows a homeostatic state of microglial cells, which can be used for better characterization (**Figure 6.3a**). Our results showed that cell density is maintained and morphological

changes such as more ramified microglia 3 days after doxycycline withdrawal (**Figure 6.3b**). The transduced microglia became more ramified IBA1+ cells and expressed higher expression of TREM2 and P2RY12 *in vitro* (**Figure 6.3b**). Another indicator that withdrawal leads to more homeostatic microglia is the relative reduction in IBA1+ baseline expression (**Figure 6.3b**). IBA1+ expression increases with age and activation state. Reduced IBA1 and increased TREM2 expression suggest more mature and homeostatic microglia. Together, these results demonstrate that transduced microglia can rapidly increase in a controlled manner and differentiate to the more mature and ramified state upon withdrawal of doxycycline.

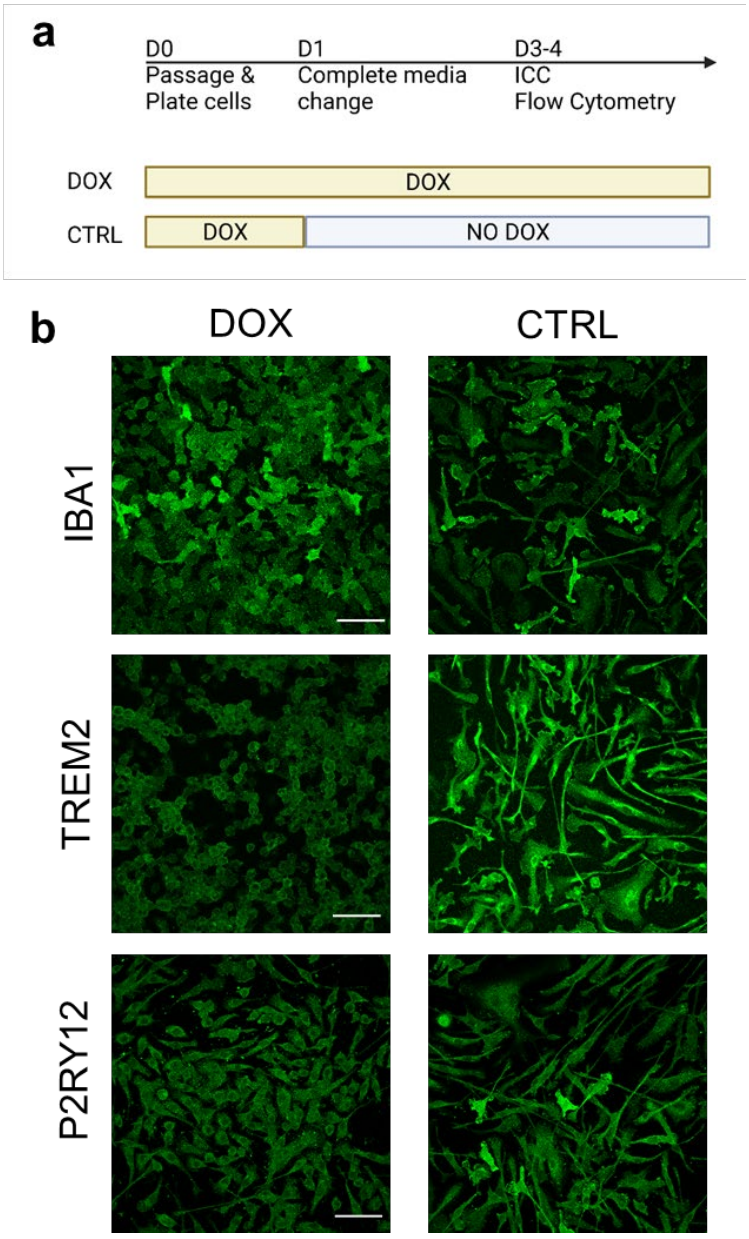


Figure 6.3. Withdrawal of doxycycline induces microglia differentiation

a) Experimental timeline for doxycycline induction (DOX) or control (CTRL) treatment groups. **b)** Representative images of (left) doxycycline induced immortalization, control (right), microglial cells at day 4. Top panel shows IBA1 expression, middle panel shows increased TREM2, and bottom panel shows increased P2RY12 expression after withdrawal of doxycycline (scale bar= 50um). Data collection, and figure by H. Yeh.

In addition to immunocytochemistry analysis, flow cytometry analysis also demonstrate doxycycline withdrawal had the most significant effect on 2E11mMG differentiation (**Figure 6.4**). Here, we compared the effect of doxycycline (DOX), macrophages colony-stimulating factor (M-CSF), and transforming growth factor-beta (TGFb) treatment. One of the most commonly used protocols for microglia culture requires M-CSF supplemental for promoting cell survival and proliferation (Smith et al. 2013; McQuade et al. 2018). M-CSF is a known inducer of PU.1 expression, increases antigen presentation protein, and enhances phagocytosis (Smith et al. 2013). In addition, TGFb is suggested to be critical for induction of homeostatic microglia signature in vitro (Butovsky et al. 2014; Bohlen et al. 2017). Thus, we assessed whether M-CSF or TGFb alters microglial phenotype in the transduced 2E11mMG cell lines. Histogram of BV421-CD45, PE-Cy7-CD11b, and APC-CX3CR1 show cell population shift toward increased CD11b and CX3CR1 expression after doxycycline withdrawal (**Figure 6.4a**). Flow cytometry of the transduced cells at 4 days after passaging revealed that, regardless of treatment, about 80% of the transduced cells express CD45 and CD11b, typical microglia markers (**Figure 6.4b-c**, left). None of the treatments significantly affected the proportion of CD45+CD11b+CX3CR1+ cells, which totaled about 30-40% of total cells across all groups (**Figure 6.4c, right**). However, doxycycline (DOX) reduced CD11b mean fluorescent intensity compared to control (CTRL), M-CSF, or TGFb treated transduced microglia (**Figure 6.4d**). This indicates the withdrawal of DOX and reduced expression of oncogenes CMYC and HRAS promotes differentiation of microglial cells.

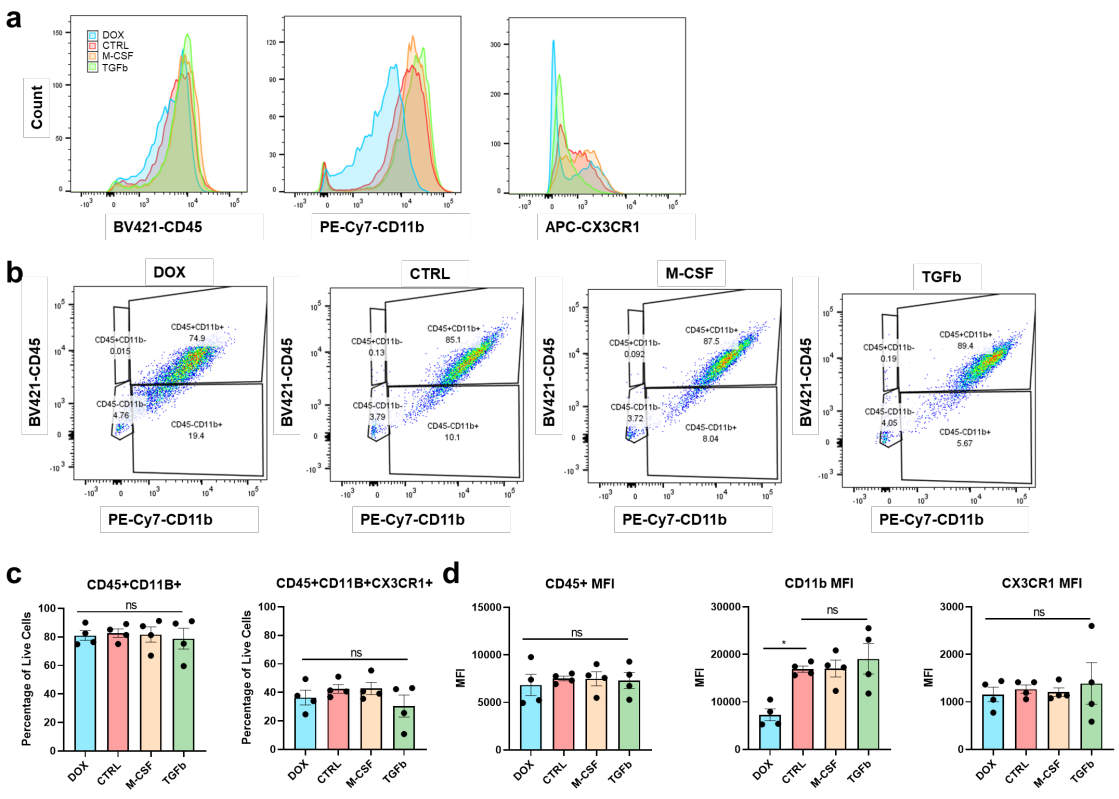


Figure 6.4 CD45, CD11b, and CX3CR1 expression of transduced microglia cells by flow cytometry

a) Histogram of CD45, CD11b, and CX3CR1 of transduced microglia treated with doxycycline (DOX, blue) or no doxycycline control (CTRL, red) or added 20ng/ml M-CSF (orange) or TGFb (green) for three days. **b)** Gating strategy for BV421-CD45 and PE-Cy7 CD11b. **c)** Percentage of CD45+CD11+ (left) and CD45+CD11b+CX3CR1+ (right) microglial cells by treatment. **d)** quantification of mean fluorescence intensity (MFI) for CD45, CD11b and CX3CR1. N=4 replicates from 2 independent batches. Statistical analysis by One-way ANOVA. *p<0.05. Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Microglia morphology can alter in response to environmental changes and immune stimulation, such as LPS stimulation (Hart et al. 2012; Ye et al. 2020; He et al. 2021). Here, we show that M-CSF treatment led to enhanced microglia process length

and increased branch points, suggesting M-CSF had a significant effect on promoting microglial differentiation and homeostatic function across all time points (**Figure 6.5a-b**). Functional alterations are associated with morphological changes in microglia (He et al. 2021). Microglia cells can secrete pro-inflammatory and anti-inflammatory cytokines and chemokines in response to immune activation such as LPS stimulation. *In vivo* study shows that LPS stimulation can activate microglia and increase microglia volume that corresponds to a neuroprotective phenotype in the context of the brain (Chen et al. 2012). Live imaging results show that LPS stimulation led to significantly increased microglia processes length at 3 hours and 24 hours post-stimulation in non-M-CSF treated control microglial (**Figure 6.5b**). These results indicate that both M-CSF and LPS alone can alter this transduced microglial morphology *in vitro*. But LPS had no significant effect on microglia morphology in the presence of M-CSF. However, M-CSF induced ramification is lower compared to *in vivo* microglia morphology with complex and greater branch complexity.

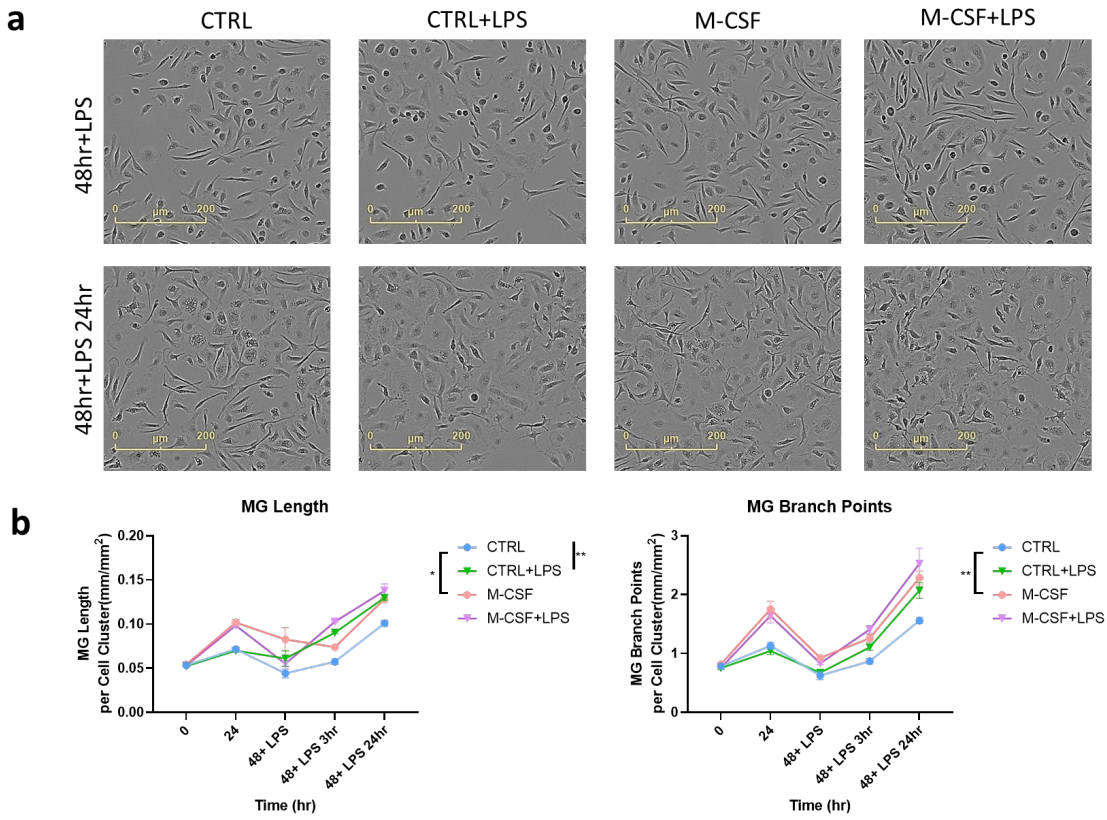


Figure 6.5 Microglia morphology altered by M-CSF and LPS treatment.

a) Representative images of microglial cells at after 3 days of treatment: withdrawal of doxycycline control (CTRL) and 20ng/ml M-CSF treatment with or without 1 ug/ml LPS stimulation at 48 h. **b**) Quantification by IncuCyte of microglia processes length and branch points at different timepoints after complete media change and M-CSF treatment. Two-way ANOVA *p<0.05, (n=6 replicates, 2 independent batches). Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Therefore, we tested whether 2E11mMG cells could secrete cytokines when stimulated by LPS after immortalization and withdrawal of doxycycline. At 3 and 24 hours following LPS stimulation, IL-6 and TNFa secretion by microglia significantly increased (**Figure 6.6a**). M-CSF treatment led to increased IL-6 secretion in response to LPS at 3 hours, but no effect at 24 hours, suggesting M-CSF can alter transiently enhance

the activation status of microglia due to LPS stimulation (**Figure 6.6a**). IL-1b and CCL2 were significantly upregulated by LPS stimulation. MCSF treatment did not affect TNFa, IL-1b, or CCL2 (**Figure 6.6a-b**). IL-10 only increased due to LPS stimulation when treated with M-CSF (**Figure 6.6b**). There was no significant batch effect except IL-10 secretion, revealing sustained microglial function regardless of passage number. In summary, LPS consistently stimulated microglial cytokine and chemokine secretion, and the transient effect of M-CSF on IL-6 and IL-10 secretion is dependent on LPS stimulation.

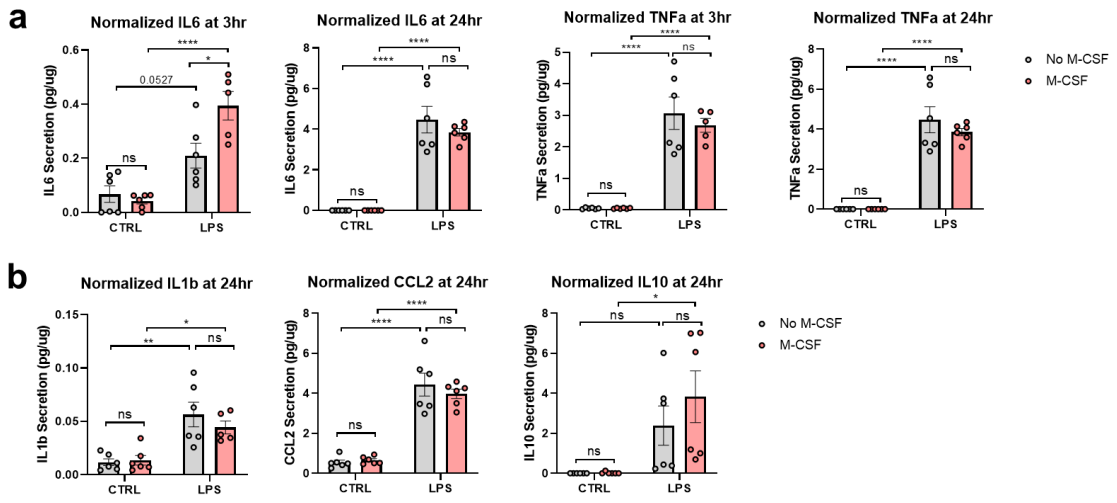


Figure 6.6 ELISA of cytokine and chemokine response to LPS stimulation of microglial cells at D3-4 following withdrawal of doxycycline.

a) IL-6 and TNFa secretion level in culture media normalized to total protein of cell lysates at 3-24 h post LPS stimulation. **b)** IL-1b, CCL2 and IL-10 secretion level normalized to total cell lysate protein at 24 h after LPS stimulation (normalized by total cell lysate protein, n=5-6 replicates, 2 independent batches). Statistical analysis by Two-way ANOVA, Tukey's posthoc multiple comparison. Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Microglia can sense and migrate toward wounded sites to repair cell damage

(Nimmerjahn, Kirchhoff, and Helmchen 2005; Davalos et al. 2005). To characterize if this inducible 2E11mMG cell line can sense and migrate to wounded sites, we performed scratch wound assay and imaged the closing of the scratch wound site (**Figure 6.7a-b**). M-CSF treatment seemed to accelerate the wound closure 12 hours after introducing a scratch wound using a pipette tip (**Figure 6.7b**). This revealed that M-CSF treatment could activate microglia to promote wound closing by increasing cell density.

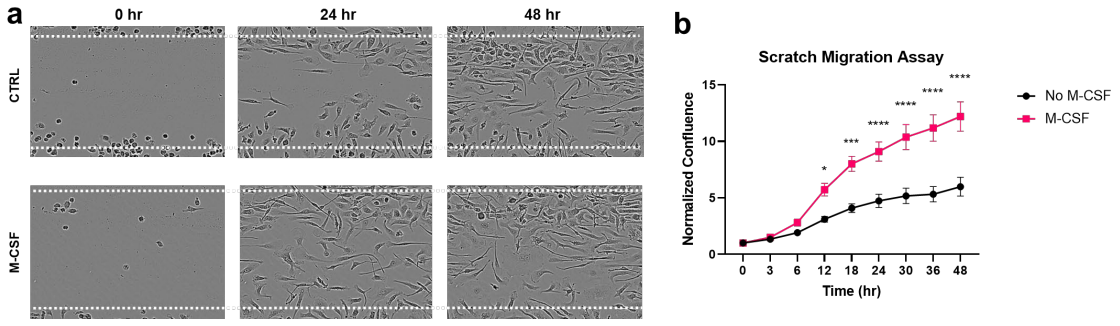


Figure 6.7. Scratch wound assay of microglial cells.

a) Representative images of control and M-CSF treated microglia cells at 0, 24, and 48 h after scratch wound. Dotted line represent track of scratch wound by pipette tip. **b)** Quantification of scratch wound migration of microglial cells over 48 h by 3 h intervals (n=4-5, 2 batches). Statistical analysis by Two-way ANOVA. Bars show mean +/- SEM. Data collection, analysis, and figure by H. Yeh.

Another typical microglia function is the phagocytosis of pathogens and cell debris. Following the withdrawal of doxycycline, the microglial cell line was treated with pHrodo *E. Coli* bioparticles to determine phagocytosis capacity. The results reveal that regardless of M-CSF treatment, the transduced microglial cells can phagocytose E. Coli bioparticles within a few hours (**Figure 6.8a-b**). M-CSF treatment had no significant effect on the total count or total integrated intensity (**Figure 6.8b**). M-CSF treatment increased the total area of pHrodo+ microglial cells at 16, 18-20 hours, suggesting

transient phenotypic changes associated with phagocytosis due to M-CSF treatment that corresponds to M-CSF enhancing microglial processes (**Figure 6.8b**). These results reveal that microglia retain the capacity for phagocytosis of *E. Coli* bioparticles after immortalization and multiple passages and do not require additional treatment with M-CSF.

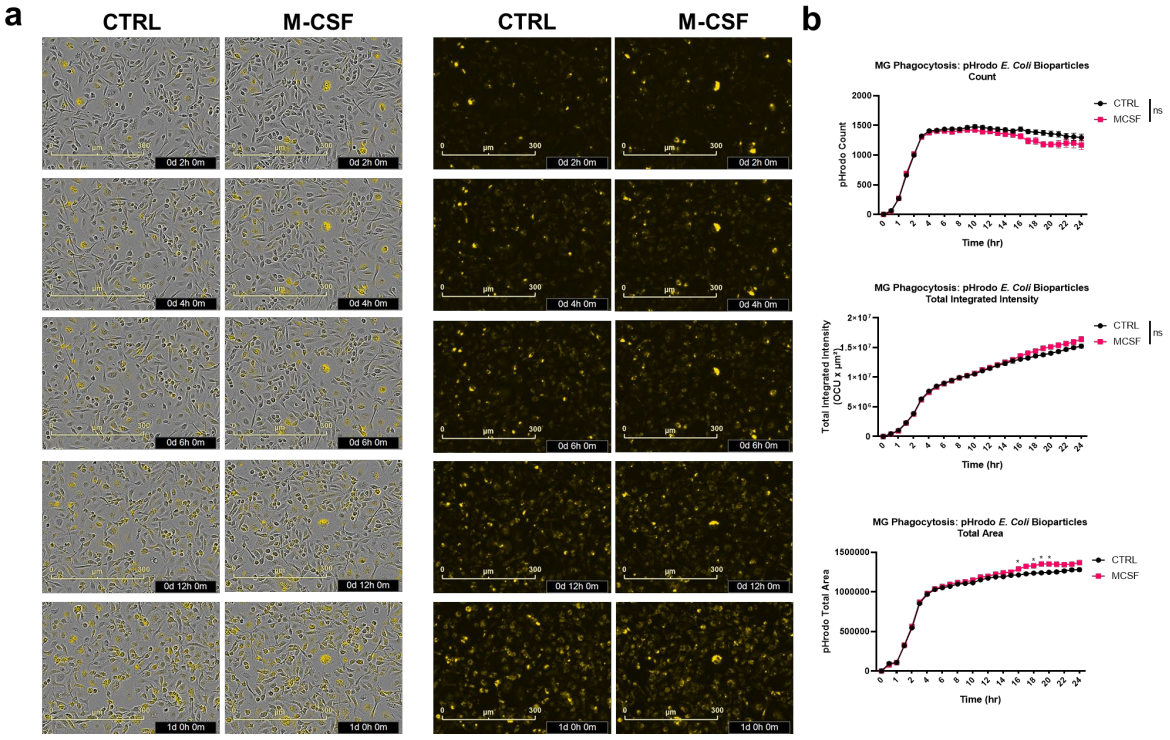


Figure 6.8 Phagocytosis assay based on pHrodo E. Coli bioparticles.

a) Representative images of merged (left two columns) and pHrodo (right two columns) at 2, 4, 6, 12, and 24 h after added E. Coli bioparticles. **b)** Quantification of pHrodo bioparticles phagocytosis including total count (top), total integrated intensity (middle), and total area (bottom). (n=6-7 replicates, 2 independent batches). Analysis by Two-way ANOVA, Tukey posthoc analysis. Data collection, analysis, and figure by H. Yeh.

6.4 Discussion

Numerous protocols have been developed to culture microglia requiring complex recipes to recapitulate microglia *in vivo* but have limited success due to the immortalization methods relying on constant expression of oncogenes (**Table 6.2,** (Stansley, Post, and Hensley 2012; Timmerman, Burm, and Bajramovic 2018; Abud et al. 2017; Butovsky et al. 2014). This study shows that inducible immortalization of primary microglial cells can lead to a more homeostatic state similar to human iPSC-derived microglial-like cells (iMGL). The induction of oncogenes CMYC T58A and HRAS G12V by doxycycline are necessary to induce rapid proliferation for CD45+CD11B microglial cell expansion. Withdrawal of doxycycline led to a more homeostatic state as demonstrated by more ramified morphology and increased TREM2 and CD11B expression. These transduced microglial cells can be passaged and maintain critical microglia functions, including secretion of cytokines in response to LPS stimulation, wound healing, phagocytosis of pathogens (**Table 6.2**).

	Primary Microglia	**BV2**	**2E11mMG**	**N9**	**HMO6**	**iMGL**
Source	Mouse	Mouse	Mouse	Mouse	Human	Human
Immortalization	N/a	Transformed v-raf/v-myc	Inducible CMYC/HRAS	Transformed v-myc	Transformed v-myc	N/a
CD11b/MAC-1	+	+	+	+	+	+
P2RY12	+→- (reduced with time in vitro)	-	+	-	N/a	+

LPS stimulation	+	+	+	+	+	+
lL-1β release	+	-	+	+	-	+
TNF- release (Following LPS)	+	+	+	+	+	+
Phagocytosis	+	+	+	+	+	+

Table 6.2. Comparison of commonly used microglia cells lines examining microglial markers and function *in vitro*. Table modified from Stansley, Post, and Hensley (2012).

Ongoing work includes transcriptomic analysis of this cell line to examine how doxycycline-induction of oncogenes CMYC T58A and HRAS G12V and growth factors M-CSF and TGFb can affect gene expression. We hypothesize that doxycycline induction leads to a more immature state that resembles PLX-treated or immature microglia precursor cells, enabling rapid proliferation. M-CSF and TGFb treatment may result in an altered state of microglia associated with a more mature phenotype. Although we did not detect strong expression of TMEM119 or P2RY12 *in vitro*, cell transplantation may restore homeostatic microglial molecular signature. The withdrawal of doxycycline may aid in differentiation but not be sufficient to increase homeostatic markers comparable to *in vitro* due to lack of critical factors such as IL-34, cholesterol, or astrocytic-derived growth factors promoting microglia differentiation (Bohlen et al. 2017). Co-culture with astrocytes or astrocytic conditioned media may further drive homeostatic gene expression and increase microglia branching. Other characterization of these transplanted microglial cells can examine neuron-glia interaction to determine how this cell line can interact with brain circuitry and prune synapses.

This inducible system was successfully implemented for mouse microglial cell lines. It would be advantageous for preclinical drug discovery to apply this method to human microglia cells, including primary microglia or iPSC-derived microglia-like cells. There are significant genetic and possibly functional differences between human and mouse microglial cells (Gosselin et al. 2017; Geirsdottir et al. 2019; Sharma, Bisht, and Eyo 2021). Expression of complement genes, such as C2 and C3, are higher in humans than mice, showing some level of differences between human and murine microglia (Gosselin et al. 2017). Human microglia remain poorly characterized due to limited human samples and lack of standardized isolation/culturing methods. Recently, protocols for human induced pluripotent stem cell (iPSC)-derived microglial-like cells (iMGLs) have been helpful in studying the biology of human microglia-like cells (Muffat et al. 2016; Abud et al. 2017; Douvaras et al. 2017; Haenseler et al. 2017; Pandya et al. 2017). These protocols differentiate iPSCs into iMGs, which have a more similar transcriptome profile compared to *in vitro* human fetal microglia than *ex vivo* microglia (Gosselin et al. 2017; Abud et al. 2017; Pandya et al. 2017). However, differentiation of microglia from human iPSC-derived cells may be costly and have significant batch effects. Thus, this inducible immortalization system may promote consistent results and be beneficial for high-throughput screening assays.

CHAPTER SEVEN

Conclusion and future directions

7.1 The role of microglia in mediating maternal immune activation

Epidemiological studies have found maternal infection as a significant risk factor for neuropsychiatric disorders, including ASD and schizophrenia (Patterson, 2009). In rodent models, MIA induces neuropathology and aberrant behaviors in domains shared by human neurodevelopmental disorders associated with maternal inflammation. MIA rodent models provide an opportunity to identify molecular targets that can mediate MIA-induced changes to neurodevelopment. More recent studies have been focused on investigating factors contributing to the susceptibility to MIA due to reproducibility issues stemming from heterogeneous and sometimes opposing findings in MIA offspring (Estes and McAllister 2016; Kentner et al. 2019; Meyer et al. 2006; Smolders et al. 2018). Poly(I:C) or LPS stimulation increases maternal serum level of IL-6 comparable to influenza administration (Meyer et al., 2006, Smith et al., 2007). However, there is a wide range of IL6 responses between different groups and varying immunogenicity of poly(I:C) (Careaga et al. 2018; Kowash et al. 2019; Mueller et al. 2019; Estes et al. 2020). Other groups have shown that a specific bacterial strain of microbiota is essential in inducing the MIA phenotype, but reproducibility has been limited (Choi et al. 2016; S. Kim et al. 2017; Yim et al. 2017; Estes et al. 2020). Previous studies have found inconsistent and often contradictory results of microglia alterations by MIA (Smolders et al. 2018). Thus, the MIA model and role of microbiota may be more complicated than previously thought. We show that our MIA mouse model using poly(I:C) induction elicits

strong IL-6 cytokine upregulation day of MIA but no changes in IL-17 serum levels. Our study focused on determining the effects of MIA on microglia since microglia is the primary cell type responsible for mediating immune response and is highly active in contributing to neuronal development. Here, we assessed behavior and microglial phenotype in the MIA offspring using various methods and the effects of CSF1R inhibition to remove and repopulate microglia in MIA brains in vivo. We found that microglia repopulation reversed MIA-induced social impairment, suggesting a critical role for microglia in mediating social behavior.

Depletion of microglia can be achieved by oral administration of CSF1R inhibitor, and removal of the small molecule led to complete recovery of microglia in both saline and MIA offspring. There was no significant difference in microglial depletion or recovery efficiency between saline and MIA. We found a transient increase in IBA1+ microglia density in the prefrontal cortex of male MIA offspring, suggesting an age-dependent effect of MIA on microglial density. The molecular mechanism of this transient increase of microglia remains unclear, but we speculate that it is related to the specific role of microglia required for each developmental period. In the mouse model, microglia continues to proliferate until the third postnatal week, which also corresponds to the continuous rise of synaptogenesis and synaptic pruning that peaks at two postnatal and stabilizes by the third postnatal week (Farhy-Tselnicker and Allen 2018; Gonzalez-Lozano et al. 2016; Kroon et al. 2019; Nikodemova et al. 2015). This suggests the need for higher microglia density to support critical periods for synaptogenesis by providing neurotrophic factors and pruning excessive synapses.

We extensively characterized microglia phenotype by morphology, protein expression of microglial genes, and transcriptomic. MIA increased microglial processes branching but this change, which was normalized by microglia repopulation treatment. The combinatorial use of P2RY12 and IBA1 enabled better characterization of microglial branching compared to IBA1+ alone. Furthermore, we found a significant reduction of CSF1R inhibitor PLX treatment on the relative intensity of IBA1 and P2RY12 protein expression by immunohistochemistry. IBA1 and P2RY12 expression increase with age as microglia matures and increases branch complexity and coverage (Elmore et al. 2018; Hammond et al. 2019). Microglia repopulation resulted in a more immature microglia state with reduced IBA1 and P2RY12 expression, suggesting epigenetic changes that restore a more homoeostatic microglia phenotype in MIA offspring. These results support the hypothesis that MIA can accelerate the maturation of microglia, leading to hyper-ramification morphology and increased microglia-neuron interaction, which can be normalized by microglial repopulation.

Further characterization of microglial transcriptome revealed upregulation of MGnD/DAM genes and downregulation of homeostatic microglial genes in Saline+MG-REP mice. More notably, MIA repopulated microglia remain homeostatic microglia, suggesting MIA microglia may respond differently to microglia repopulation. Fate mapping by BrdU/EDU labeling was performed to label proliferating microglial cells before and after the depletion period to characterize the origin of repopulated microglia. Our study suggests two distinct microglial populations for expansion after CSF1R inhibitor withdrawal in MIA offspring. We show that a higher proportion of MIA

microglia repopulates from the non-proliferative pool, whereas a higher proportion of Saline microglia repopulates from the remaining proliferative microglia pool. One possible explanation for this phenomenon is that MIA MG tends to have accelerated maturation, leading to premature microglia expansion before BrdU labeling during the postnatal period. One limitation of this method is the relatively inefficient labeling of proliferating microglial cells. Microglia can rapidly expand within a few days and have a limited window for labeling (Elmore et al. 2014; Huang et al. 2018; Zhan et al. 2020), thus reducing labeling efficiency.

Further analysis of microglia transcriptome revealed MIA increases neuritogenic molecules, which support neurite growth and plasticity. Many studies have shown that microglia can express and secrete neurotrophic factors such as BDNF or IGF to promote proper neurodevelopment and synapse formation (Parkhurst et al. 2013; Ueno et al. 2013). Another study shows phagocytosis of apoptotic cells induced microglial expression of neurogenesis genes related to neuropeptides such as VGF, trophic factors such as VEGF, and FGF2, matrix metalloproteases, and cytokines (Diaz-Aparicio et al. 2020). This is the first study to identify MIA-induced overexpression of neuritogenic/neurogenic genes that can lead to hyperconnectivity and be normalized by microglial repopulation. We selected a subset of select neuritogenic molecules to validate and rule out contamination as a potential source of neurogenic molecules and confirmed MIA-induced upregulation using ISH, qRT-PCR of FACS-isolated microglia, and ELISA. NCAM2 and WNT5a were consistently increased by MIA across various methods of validation. We identified key molecules that can be potentially targeted for

understanding the MIA effects on microglia-neuron interactions. Together with previous studies, this study demonstrates strong evidence that microglia can express neuritogenic or neurotrophic factors depending on developmental age and brain environment. We show that maternal inflammation can upregulate the expression of neuritogenic genes.

Thus, we examined neuronal physiology and morphology using whole-cell patch-clamp and immunohistochemistry. MIA increased intrinsic excitability and dendritic spine density in Layer V IB neurons of mPFC, suggesting altered neural circuitry due to maternal inflammation. Further examination of microglia-neuron interactions revealed enhanced microglial interactions with neurons due to MIA, which was corrected by microglial repopulation. To determine if MIA microglia can promote dendritic spine density, we co-cultured saline or MIA microglia with control neurons and imaged spine density *in vitro*. We found an increased proportion of dendritic spines that are filopodia spines, indicating MIA MG promotes the growth of immature spines, possibly via the secretion of neurogenic molecules. The spine densities were higher in layer II pyramidal and layer V in the temporal lobe of ASD patients (Hutsler and Zhang, 2010). Soumiya et al. also found that MIA increases the dendritic spine density in adult MIA offspring and decreased synaptophysin- and glutamic acid decarboxylase-67- positive puncta surrounding the neuronal cell bodies in the layer II-III neurons (2011). Another study, however, revealed reduced dendritic spines densities of Thy1-YFP+ layer V neurons in P30 MIA offspring (Coiro et al. 2015). One explanation is the difference in timing of poly(I:C) injection, in which Coiro et al. induced MIA at a later timepoint at E12.5 that could target other cell types since the initial microglial migration to CNS started at E9.5.

Another plausible explanation for this discrepancy was that we also observed transiently reduced mRNA expression levels of microglial neuritogenic factors at P20, comparable age to Coiro et al's study when they observed synaptic deficits. This critical period corresponds to the upregulation of inflammatory cytokines in MIA offspring, further suggesting that inflammatory conditions may regulate aberrant microglial functions (Garay et al., 2013). Overall, these findings support our hypothesis that MIA enhanced microglial secretion of neuritogenic molecules and contribute to increased neuronal excitability and hyperconnectivity. Thus, microglia play a critical role in mediating MIA-induced deficits, which can be ameliorated by microglial repopulation.

In addition to MIA-induced upregulation of neuritogenic molecules in MIA microglia, we found MIA-induced downregulation of genes associated with immune response and complement pathways, which were normalized by microglial repopulation using RNA-seq. Mattei et al. showed that microglial phagocytosis is impaired by MIA and rescued by anti-inflammatory minocycline treatment (2017). Interestingly, some level of microglia activation is required for layer V pyramidal neuronal survival as minocycline treatment resulted in increased cell death of layer V neurons (Ueno et al. 2013). Therefore, future studies are required to assess if mediating the inflammation status of microglia can directly drive neuritogenic MIA phenotype and secrete neurotrophic factors. It also remains unclear whether reducing neuritogenic gene expression can correct MIA-induced deficits. We can test this using microglia conditional deficiency of neuritogenic genes such as NCAM2 or WNT5a in MIA offspring to

determine if reducing NCAM2 or WNT5a expression in microglia can alleviate social impairment and neuronal hyperexcitability.

Future studies are required to assess and validate if MIA microglia are deficient in synaptic pruning and whether microglia repopulation can reverse pruning deficits. Synaptic deficits can be targeted by using approaches that can promote synaptic pruning pathways, such as promote microglial expression complement component 3 by neuronal exosomes stimulation or reducing CD47 signaling pathway that prevents neuronal synapse elimination by microglia-specific deletion of SIRPα (Bahrini et al. 2015; Ding et al. 2021). In addition to examining layer V pyramidal neurons, other regions such as layer II and hippocampal neurons can be assessed for the effect MIA and treatment for modulating synaptic pruning by exosome addition or microglial-deletion of SIRPα since these regions are also implicated in neurodevelopmental disorders (Velmeshev et al. 2019; Pekala, Doliwa, and Kalita 2020). According to our gene ontology of MIA microglia, MIA reduced the expression of genes associated with the complement pathway. Thus, promoting synaptic pruning is another approach in alleviating MIA-induced deficits in neuron physiology and behavior.

Of note, microglia repopulation in saline offspring led to unexpected alterations in neuronal properties similar to MIA-induced enhanced excitability, suggesting an altered supportive role of repopulated microglia. This corroborates previous studies showing a homeostatic microglial role in providing negative feedback to prevent excessive neuronal activity (Liu et al. 2020; Badimon et al. 2020). Maternal inflammation led to disrupted excitation and inhibition balance in the brain by promoting an alternate state of microglia

that enhances neuronal excitability. Further work is required to examine if other cell types are also affected by MIA and microglia repopulation, such as using single-cell RNA-seq. Other cell types to be assessed can include oligodendrocyte precursor cells and astrocytes, which can also regulate synaptogenesis and refining brain circuitry (Buchanan et al. 2021; Jang et al., 2019.; Eroglu and Barres 2010). Furthermore, oligodendrocyte precursor cells show low expression of CSF1R, which may be affected by PLX5622 CSF1R inhibition administration (Zhang et al. 2014). On the other hand, astrocytes may also compensate for microglia loss during the microglia depletion period as there is an increased mRNA and protein expression of GFAP and S100b, although no changes in morphology or cell density of astrocytes were observed (Elmore et al. 2014). But determining how other glia cells interact with microglia and neurons can better understand the effect of MIA and microglia repopulation treatment on the brain circuitry.

In our study, microglia characterization has been focused on male offspring because of the increased susceptibility to neurodevelopmental disorders and MIA. Most MIA studies have found a more robust MIA phenotype in males. But more recent studies have revealed sexual dimorphisms of microglial response to immune challenge and microbiota composition changes (Thion et al. 2019; Bordeleau et al. 2019; Hui et al. 2020). Thus, examining sexual differences may be critical in understanding the increased male susceptibility for neurodevelopmental disorders. Ongoing work includes exploring sex differences in microglia depletion and repopulation of saline and MIA offspring.

This study shows CSF1R inhibition as a promising therapeutic strategy for neurodevelopmental disorders, including autism spectrum disorders in which social

deficits and increased neuronal excitability are observed. MIA has been shown to alter gut microbiota and increase peripheral inflammation (S. Kim et al. 2017; W. Li et al. 2021). CSF1R inhibition can have significant effects on peripheral inflammation and immune cells. Hence, investigating whether CSF1R inhibition may reduce peripheral inflammation or mediate cytokine release from gut macrophages in MIA offspring can reveal the role of peripheral immune cells in MIA. CSF1R inhibition by PLX5622, as used in our study, can potentially cause long-term changes in hematopoiesis and the function of macrophages (Lei et al. 2020). Thus, additional dosing and toxicology studies may be required to assess the risk and benefit of using CSF1R inhibition for treating neurodevelopmental disorders.

7.2 Microglia WNT5a implicated in mediating synaptic maintenance

Wnt signaling is emerging as a key regulator of microglia phenotypes and mediating the interaction between neurons and microglia. Microglia can secrete Wnt ligands depending on their activation status and mediate neural stem cell differentiation and oligodendrogenesis (Mecha et al. 2020). LPS promoted the microglial secretion of WNT5a, whereas IL4/IL13 stimulation enhanced microglial secretion of WNT7a and promoted oligodendrogenesis of neural stem cells (Mecha et al. 2020). WNT3a activation of Wnt/β-catenin signaling resulting in β -catenin accumulation or activation of noncanonical Wnt5a pathway can induce pro-inflammatory microglial responses *in vitro* (Halleskog and Schulte 2013; Halleskog et al. 2011). Thus, both canonical and non-canonical Wnt pathways can be further evaluated to determine how Wnt pathways can

mediate microglia phenotype and function during development and neurological disorders.

To determine how microglial secretome can affect neurodevelopment, we performed microarray analysis to assess the transcriptomic changes in neural stem cells treated with microglia conditioned media. We found microglia secretory factors led to more mature and differentiated neural stem cells. WNT pathways were altered by microglial conditioned media, possibly via binding to ROR receptors. We show that WNT5a is the most abundantly expressed Wnt ligands in homeostatic and activated microglia. WNT5a was one of the key MIA-induced neuritogenic molecules (Ikezu et al. 2020). Thus, further examination was focused on assessing the role of WNT5a in microglia. In mixed glial culture, we found WNT5a predominately expressed by microglial cells compared to oligodendrocytes or astrocytes. There is an age-dependent expression of WNT5a, suggesting its primary involvement during early development. Of note, in human cells, microglia were the primary source of WNT5a.

To further elucidate the role of MIA-associated WNT5a in microglia, we used siRNA silencing and developed a co-culture for neurons and microglia. We found that microglial Wnt5a can be efficiently silenced by siRNA transfection with minimal effect on microglial phenotype. qRT-PCR results confirmed significant increased WNT5a and C1qa expression and increased IL1b expression. Phagocytosis capacity was not significantly altered by silencing of WNT5a. We assessed the effect of silencing efficiency using siRNA transfection in the context of primary microglia culture. Thus, understanding if silencing WNT5a persists in co-culture conditions may be critical.

Silencing genes in microglia specifically has been challenging due to the nature of microglial cells, which resist transfection and transduction and often result in massive cell death. Lentiviral shRNA silencing WNT5a has been tested but with no success. Further assessment is necessary to determine if silencing WNT5a can affect other WNT pathways and microglial genes. Microglial-conditional knockout of WNT5a mouse model can be assessed for microglia-specific knockout of Wnt5a and determine if other Wnt pathway genes are affected. To validate the importance of microglial WNT5a, other Wnt genes expressed by microglia, such as WNT7a and Wnt3a, can also be conditionally reduced in a mouse model to compare whether phagocytosis and neuron-microglia interaction are altered.

Microglia critically mediate neurodevelopment and promote neuronal differentiation. We found that microglia treatment to primary Thy1-YFP cortical neurons enhanced neuronal outgrowth and dendritic spine density. In addition, silencing microglial WNT5a led to diminished neurite length and complexity similar to neurons without microglia, suggesting a critical role of microglial WNT5a in neurite outgrowth. Further analysis of dendritic spine density and turnover revealed that microglia increased Thy1-YFP spine density and reduced the spine turnover rate. In addition, microglial WNT5a promotes spine stabilization by reducing spine appearance and elimination, indicating increased spine maturity and stabilization.

Previous studies show that microglial contact can induce Ca2+ influx and promote filopodia extension (Akiyoshi et al. 2018; Weinhard et al. 2018). Here, we report an alternative mechanism for microglia to promote neurite extension and spine growth

via secretory molecules, including Wnt5a. BDNF, another neurotrophic factor secreted by microglia, is a direct target of the canonical Wnt pathway in glial cells (Yi et al. 2012). Other studies have shown that IL-10 secreted from microglia can also affect synapse formation by increasing dendritic spine number (Lim et al. 2013). This would indicate that WNT5a is not the only factor affecting neural circuitry remodeling in vitro. Therefore, to test this hypothesis, we can add neutralizing antibodies to deplete different cytokines or microglial secretome factors and determine neurite and spine development. Further examination of synapse density by immunocytochemistry revealed microglia increases PSD95 and synapse colocalization of VGLUT2 and PSD95. These results show microglia can increase synapse density via a post-synaptic mechanism, consistent with previous studies demonstrating that neuronal WNT5a acts on maintaining dendrite architecture and post-synaptic assembly via the Ca2+ pathway (Chen et al, 2017). Validation of physiological relevance is necessary to determine if microglial WNT5a can impact brain development *in vivo*. Knockout of WNT5a has been challenging to achieve due to its role in embryonic development, thus conditional deletion of WNT5a in microglia is necessary. Synaptic molecules and synapse density can be examined in microglia-specific WNT5a deficient mice. We hypothesize reduction of microglial WNT5a may impact synapse density in a region-specific manner. WNT5a expression is also dependent on brain region and neuronal subtype, as WNT5a expression is increased in hippocampal CA1 neurons, layer II/III and V pyramidal neurons, and inhibitory SNCG/VIP GABAergic neurons (Yao et al. 2021). Another possibility is based on expression of WNT5a receptors, such as ROR1/2, which is upregulated in inhibitory

SNCG/VIP GABAergic neurons. Thus, inhibiting microglia WNT5a may be greater impact in neurons with enrichment of WNT5a pathway or higher expression of WNT5a receptors.

Our study explored the role of WNT5a in embryonic microglia and its impact on normal early development. The expression and function of Wnt5a are dependent on age and disease context (Ikezu et al. 2020; Florian et al. 2013). Basal Wnt5a has a synaptoprotective effect against the initial amyloid-beta via preventing amyloid-beta-induced impairment on post-synaptic structure (Li et al. 2011; Varela-Nallar et al. 2012; Cerpa et al. 2010). Thus, it would be interesting to investigate whether silencing microglial WNT5a can alleviate MIA-induced deficits or mediate Alzheimer's disease pathology. Excessive microglial Wnt5a is expected to be detrimental in the MIA model due to increased neuronal excitability, whereas it could be beneficial in the context of aging and neurodegeneration due to diminished expression of WNT5a with age (Ikezu et al. 2020; Zhang et al. 2014). Increasing microglial WNT5a is expected to prevent synapse loss and strengthen neuronal connectivity. This opposing effect in context of disease show maintaining normal WNT5a expression level based on age and context could be critical in preventing excessive neuritogenesis/synaptogenesis during development and synapse loss in neurodegeneration.

7.3 Developing inducible immortalization of a murine microglial cell line to investigate more homeostatic microglial functions

Primary microglia can be laborsome and costly with significant batch effects, making data interpretation difficult due to high variability between batches. Following extensive optimization and testing various oncogenes and small molecule approaches, we developed an inducible immortalized microglia cell line using tetracycline-controlled transcriptional activation with oncogenes CMYC T58A and HRAS G12V. This inducible system is more physiological relevant compared to currently available cell lines such as BV2, which resembles activated microglia than homeostatic microglia. In addition, we generated multiple monoclonal microglia cell populations by FACS and expanded single-cell colonies, thus guaranteeing the homogenous and high purity of the microglial cell population.

Following confirmation of successful transduction and induction of CMYC and HRAS by doxycycline treatment, we characterized the cell line by flow cytometry, immunocytochemistry, cytokine profiling, phagocytosis assay, and scratch-wound healing assay. These results show that the transduced microglial cell line can resemble homeostatic microglia with migratory and phagocytosis capacity after doxycycline withdrawal. Ongoing work includes RNA-sequencing of the monoclonal microglial cell line under different treatments to assess whether M-CSF or TGFb treatment can promote homeostatic gene expression comparable to ex vivo or in vivo microglial cells. Furthermore, it would be interesting to investigate the common mechanisms between activation of CMYC/HRAS and repopulated microglia by CSR1R inhibition. Withdrawal

of doxycycline led to a stable cell population and eventual cell death, suggesting limited self-renewal capabilities in homeostatic microglia.

Despite increased ramification and differentiation of microglia following doxycycline withdrawal, the transduced microglial cell line has a weak expression of typical homeostatic microglial markers such as *Tmem119* and *P2ry12*. Compared to the commonly used BV2 cells, the 2E11mMG inducible immortalized microglial cells show more ramification and a more homeostatic profile as demonstrated by the expression of P2RY12 and IBA1. This is consistent with Bennet et al. findings that prolonged exposure to in vitro environments reduces homeostatic microglia expression. Thus, ongoing work also includes determining if transplantation of the transduced microglial cells can regain more homeostatic function and integrate with brain circuitry *in vivo*.

This study provides a valuable tool to study microglia *in vitro*, enabling a more efficient method to investigate gene and drug targets. The inducible immortalization approach allows rapid expansion of monoclonal microglial cells, and doxycycline withdrawal induces a more homeostatic phenotype and functions. However, compared to human iMGL cells, the 2E11mMG cells can be further improved to resemble *in vivo* human microglia by additional growth factors and avoidance of serum that could prevent differentiation of microglial cells. Future work can apply this immortalization method to human microglial cells, either acute isolated human microglial cells from fetal or postmortem tissue or iPSC-derived microglial cells. Gosselin et al. reported that most human and mouse microglia transcript levels were similar, but many genes have a species-specific bias in expression magnitudes (2017). Thus, testing in both human and

mouse microglial cells provides a more comprehensive and physiological-relevant understanding of microglial function.

In summary, microglia can mediate the effects of maternal inflammation and alter brain circuitry and behavior outcome. During the developmental period, microglia are susceptible to immune challenge, which can have a long-lasting impact on the microglial profile and microglia-neuron interaction. Microglia depletion and repopulation can alleviate MIA-induced impairments in behavior, neuronal hyperexcitability, and associated upregulation of neuritogenic/neurogenic genes in microglia. We further characterized one of the neuritogenic/neurogenic genes, Wnt5a, in microglia and found microglial Wnt5a can maintain synaptic spine stability and enhance neuronal firing activity *in vitro*. We also developed an inducible mouse microglia cell line to understand *in vitro* microglia biology better. This inducible system can allow scientists to overcome limited biology samples and batch effect issues. Using this system, we can further test additional neuritogenic/neurogenic genes and how inflammation can alter microglia-neuron interactions.

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