Neuritogenic function of microglia in maternal immune activation and autism spectrum disorders

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Autism spectrum disorder and maternal immune activation: Environmental factors during pregnancy, such as infections, maternal stress or autoimmune disorders, are closely associated with the prevalence of neurodevelopmental disorders including autism spectrum disorder (ASD), bipolar disorder and schizophrenia. It has been shown that severe infections during pregnancy cause maternal immune activation (MIA) and significantly increase the risk of ASD in the offspring although the mechanisms are poorly understood. Many rodent MIA studies support this causal link by showing that offspring of dams administered with polyinosinic:polycytidylic acid (polyI:C), a viral mimetic Toll-like receptor 3 agonist, exhibit long-lasting ASD-like behavioral abnormalities such as increased repetitive behavior, impaired social interaction and communication. Interestingly, MIA alters inflammatory cytokine expressions persisting through development and adulthood in the brain of the offspring, suggesting that chronic neuroimmune dysfunction plays a role in mediating the deleterious effects of MIA on neurodevelopment (Garay et al., 2013).

Microglial role in synaptogenesis: Recent studies suggested that microglia, the primary innate immune cells in the brain, are potential key players in mediating the effects of MIA-induced neuroinflammation and abnormal behaviors (Mattei et al., 2017). Detailed microglial influence on the MIA phenotype in the offspring is yet to be elucidated. Microglia are sentinel cells, act as the first responders to brain insults and provide clearance and regeneration of neuronal cells. They are also critical in brain development and maturation, such as clearing apoptotic cells, supporting neurogenesis and neuronal circuitry through the synaptic formation and pruning (Miyamoto et al., 2016). Microglia migrate from the yolk sac and infiltrate into the developing brain around E9.5 in mice. Their number in the brain dramatically increases in early postnatal phase, reaching a maximum by postnatal day 18. Synaptogenesis in mouse cortical neurons accelerate during the first postnatal week and reaches to a maximum level by postnatal day 30. It is widely believed that migrated microglia actively support the early synaptogenesis through their interaction with neurons. Deficiencies in microglial neuritogenic molecules, brain-derived neurotrophic factor, results in deficits in learning-related synapse formation. Deletion of microglial phagocytic genes, such as fractalkine microglial receptor (Cx3cr1) or progranulin, leads to altered synaptic pruning and neural circuitry, and enhancement of repetitive behavior. These studies suggest that the intricate positive and negative regulation of synaptic network by microglia is critical for brain development and functions.

Neuritogenic function of microglia in MIA: In our recent paper, "*Inhibition of colony stimulating factor 1 receptor (CSF1R) corrects maternal inflammation-induced microglial and synaptic dysfunction and behavioral abnormalities*" (Ikezu et al., 2020), we uncovered a novel role of microglia in the pathogenesis of MIA and identified microglia as a promising therapeutic target. In this study, we depleted and repopulated microglia in MIA offspring via a pharmacological inhibition of CSF1R during the juvenile period following MIA induction at E9.5 to determine the effect of immune perturbed microglia on offspring development. Strikingly, depletion and repopulation of microglia ameliorated MIA-induced abnormal behaviors, including social deficits and repetitive behavior, as determined by social interaction and self-grooming tests at P60. To understand the underlying molecular changes of microglia by MIA, we performed RNA-sequencing of freshly isolated microglia from the brain of control (from saline-injected pregnant dams) and MIA (from poly I:C-injected pregnant dams) offspring at E17, P7, P20 and P60, as well as from P60 control or MIA offspring fed with chow containing CSF1R inhibitor between P21–42, which were analyzed by Database for Annotation, Visualization and Integrated Discovery (DAVID) (**Figure 1A**). Interestingly, the gene expression pattern of MIA microglia isolated at P60 showed enhanced "synaptogenic" properties. DAVID Gene Ontology (GO) biological process analysis revealed that the most enriched GO terms upregulated by MIA and corrected by microglial repopulation at P60 were "synaptic vesicles" and "neurotransmission", while the most enriched GO terms down-regulated by MIA and corrected by microglial repopulation were "antigen presentation", "complement activation" and "innate immune response." MIA microglia are thus synaptogenic, while repopulated microglia restored their innate immune functions. MIA may also have an impact on how microglia repopulate: repopulated control microglia possessed a robust immature microglia phenotype, characterized by upregulation of molecules related to phagocytosis, chemotaxis and proliferation, also reminiscent of disease-associated/neurodegenerative microglia. In contrast, repopulated MIA microglia showed an enhancement of homeostatic phenotype, characterized by upregulation of *Tmem119* and *Cx3Cr1*, suggesting maternal inflammation accelerated microglia maturation that persist through microglia repopulation. Acceleration of microglial maturation in MIA offspring was also reported previously (Matcovitch-Natan et al., 2016). Additionally, these MIA microglia repopulated mainly from previously non-mitotic (bromodeoxyuridine negative) microglia, which may revert to a non-proliferative gene expression phenotype similar to homeostatic microglia. In accordance with the GO term analysis, Ingenuity Pathway Analysis of microglial gene expression revealed upregulation of "neuritogenic" factors in MIA offspring, which were corrected by microglial repopulation. This enriched neuritogenic gene expression was most prominent at E17, which was validated by *in situ* hybridization in the cortical plate of the medial prefrontal cortex (mPFC) in male MIA offspring for catenin delta 2 (*Ctnnd2*), a synaptogenic factor, neuronal cell adhesion molecule 2 (*Ncam2*), an enhancer of filopodia formation, pleiotrophin, a secretory growth factor (*Ptn*) and wingless-type MMTV integration site family member 5A (*Wnt5a*), an axon guidance and synaptogenic molecule. Consistent with the results from the RNA-sequencing, the protein levels of *Ctnnd2*, *Ncam2* and *Ntrk2* (neurotrophic receptor tyrosine kinase 2) were upregulated in microglia isolated from MIA offspring at P60 compared to Saline controls, which was reversed by microglial repopulation. Aberrant neurosupportive function by microglia after their acute inflammatory activation has been previously reported in the rodent models of epilepsy (Choi et al., 2008) and ischemia (Madinier et al., 2009). After epileptic or ischemic events, microglia aberrantly produced proliferative or synaptogenic factors, such as insulin like growth factor-1 or brain-derived neurotrophic factor, however, the role of such factors secreted from microglia on disease progression remains unclear. Could exacerbated neuritogenic factors secreted from MIA microglia be detrimental during the crucial period of neural development when precise programming for neural circuitry is in place? Indeed, similar phenomena have been observed in ASD patients with somatic gene mutations. ASD-linked gene mutations such as *FMR1*, *PTEN*, *EIF4E* and *CYFIP* were closely related to increased immature synapse formation and altered synaptic strength via increased gene transcription and protein translation. Interestingly, FMRP-targeted genes *Ctnnd2*, *Enc1*, *Ntrk2*, *Ntrk3* and *Ryr2* were also found to be enriched in our MIA microglial gene expression analysis. These changes in MIA microglia transcriptome and proteome indicate that MIA microglial function may be driven towards increased synaptogenesis (**Figure 1B**), which can be reversed by therapeutic microglial repopulation.

Aberrant microglia-synapse interactions in MIA: To understand how these transcriptomic and proteomic changes in MIA microglia are reflected in MIA offspring, we investigated the effect of MIA on synaptic density and microglial interactions with pre- and post-synaptic neuronal structures in the layer V of the mPFC at P60. For this purpose, we examined the dendritic spines of biocytin-filled intrinsically bursting neurons in the layer V of the mPFC, whose electrophysiological properties were significantly altered after MIA by showing slower action potential kinetics and reduced spontaneous excitatory postsynaptic currents. Notably, total and filopodia (labile, immature dendritic spines that may develop into mature spines) spine densities were significantly increased in MIA compared to the control adult offspring. Confocal imaging and three-dimensional reconstruction analysis of MIA microglia revealed increased complexity in microglial branching, which were interacted with spines in closer proximity in MIA compared to control adult offspring. Most importantly, the changes caused by MIA seem to be plastic, since microglial depletion and repopulation either normalized or negated the effect on spine and microglial morphology and their interaction. Taken together, these data indicate the pathological function of microglial neuritogenic factors and consequent spine dysgenesis and aberrant microglial-synapse interaction. Further investigation is required to understand causal effect of these changes on MIA abnormal behavior.

Relevance to other MIA studies: Finally, we cross-examined our results with other studies related to MIA or microglia biology. There is evidence to support our finding for the increased spine densities. Soumiya et al. (2011) also found that prenatal polyI:C injection decreased synaptophysin- and glutamic acid decarboxylase-67- positive puncta surrounding the neuronal cell bodies in the upper-layer frontal cortical neurons and increased the dendritic spine density in postnatal 8-weeks-old offspring. Additionally, evidence from postmortem ASD human brain tissue showed an increase in spine density on apical dendrites of pyramidal neurons from cortical layer II in frontal, temporal and parietal lobes and layer V in the temporal lobe, which was inversely correlated with cognitive function (Hutsler and Zhang, 2010).

Recent studies support the notion that microglial interaction with spines may facilitate spine formations. Microglial contacts to dendritic shafts induced filopodia formation (Weinhard et al., 2018). Miyamoto et al. observed higher filopodia formation rates by microglial contact compared to non-contact sites, and maturation of microglia-induced filopodia to functional excitatory synapses (Miyamoto et al., 2016). These previous studies may explain our findings that complexed morphology of microglia and their increased interaction of spines led to aberrantly increased spine densities in MIA offspring. Finally, aberrant neuritogenic factors produced in MIA microglia in our study have been reported to affect spine formation. For instance, *Ncam2* is expressed in both neurons and microglia, and could trigger intracellular Ca^{2+} elevation that may subsequently recruit actin and elicit filopodia formation (Sheng

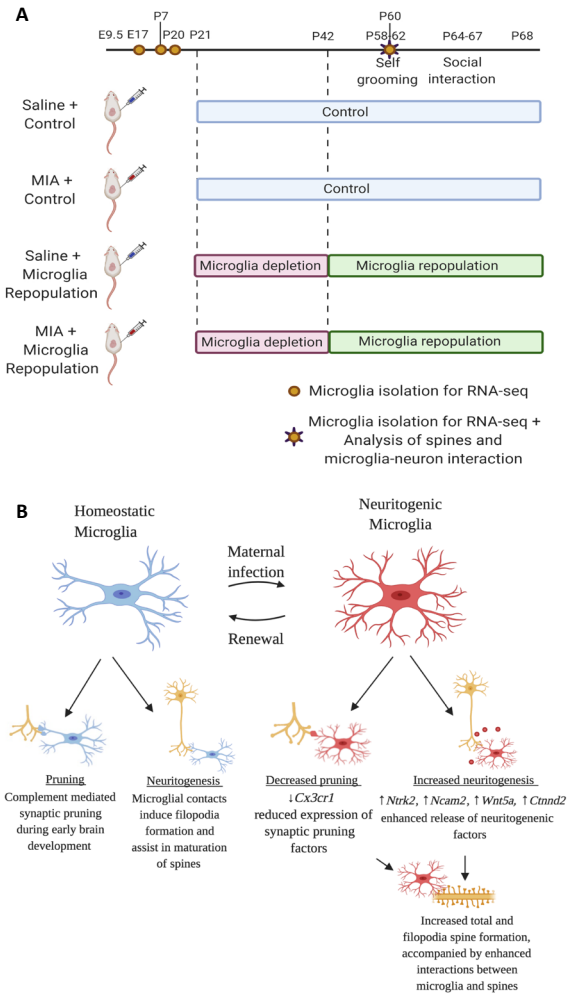

Figure 1 | Experimental design and changes in microglial function due to maternal immune activation.
(A) Timeline of drug treatment, behavioral tests, microglia isolation for RNA-sequencing and analysis of spines and microglia-neuron interaction. Pregnant dams were administered PolyI:C or Saline at embryonic day (E)9.5 followed by microglial depletion from post natal day (P)21–P42 and microglial repopulation from P42–P68 in their offspring. (B) Schematic diagram depicting changes in microglial function due to maternal immune activation. The normal physiological functioning of microglia involves complement mediated synaptic pruning and neuritogenesis by induction of filopodia formation and assisting in spine maturation (Miyamoto et al., 2016). Following maternal immune activation, microglia have increased release of neuritogenic factors (Ikezu et al., 2020) and reduced expression of synaptic pruning factors (Fernández de Cossío et al., 2017). As a result, the MIA microglia have increased interactions with spines which coincides with increased total and filopodia spine formation (Ikezu et al., 2020).

et al., 2015). *Wnt5a* has been found to stimulate dendritic spine morphogenesis and induce de novo formation of spines (Varela-Nallar et al., 2010).

Another study, however, revealed reduced synaptic densities in MIA offspring. *In vivo* multiphoton imaging in the cortex of young MIA offspring showed a reduction in number and turnover rates of dendritic spines, which persisted into adulthood (Coiro et al., 2015). The discrepancy in results could be explained by the difference in the time frame of MIA induction between their model (E12.5 poly I:C injection) and our model (E9.5 poly I:C injection, the critical time period when microglia migrate from the yolk sac). Another plausible explanation for this discrepancy can be the reduced expression levels of microglial neuritogenic factors at P20, around the time frame when Coiro's study found a reduction in spine densities, while those were upregulated at E17 and P60 in MIA compared to control offspring according to our microglial RNA-seq data. This is reminiscent of the periodical change in cytokines in the cortical region observed in MIA offspring (Garay et al., 2013), further suggesting the possibility that inflammatory conditions may regulate microglial aberrant functions. Deficits in microglial pruning are also thought to play a role in increased synaptic densities. Induction of MIA by bacterial lipopolysaccharide revealed that the offspring had a significant increase in spine number in the dentate gyrus accompanied by reduced hippocampal expression of CX3CR1, which is involved in pruning (**Figure 1B**) (Fernández de Cossio et al., 2017). A recent study also found that MIA microglia were less phagocytic as determined by microglial phagocytosis and transcriptome analysis (Mattei et al., 2017). We do not know yet how pruning deficits may play a role in MIA models or MIA-mediated ASD. According to our microglial RNA-seq data, C1q but not C3 or C4 complement complex genes were affected by MIA during development. More importantly, the symptoms of ASD appear at an early age, such as 12–18 months old, when synaptogenesis is still being progressed and synaptic pruning is less active. Bosl et al. (2018) were able to differentiate patients who would develop ASD as early as 3 months of age from non-symptomatic volunteers by analyzing their EEG measurements. As such, it is a plausible hypothesis that the increased neuritogenic factors from MIA microglia could induce increased spines and synaptogenesis in MIA-mediated ASD, which can be detected in cerebrospinal fluid or blood as future biomarkers for ASD.

In summary, our findings of aberrantly increased neuritogenic factors from MIA microglia during neurodevelopment may shed light on the molecular mechanism of microglia-mediated MIA phenotype in the offspring, such as increased synaptogenesis found concurrently with behavioral deficits in the form of increased repetitive behavior and impaired social interactions, which may be translatable to MIA-related ASD. Additionally, depletion and repopulation of microglia could be a potential therapy for MIA-related neurodevelopmental disorders.

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