Sex-Specific Impacts of Maternal Stress on the Fetal Microglial Phenome

The prevalence of neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD), has increased dramatically over the past two decades, with growing evidence linking prenatal stress to these conditions (6,7). A key feature across various maternal stressors is immune system dysregulation, suggesting that fetal microglia, may detect changes in the intrauterine environment and respond by altering neurodevelopment (4). Additionally, research has shown differences in microglial responses in males and females, which is particularly relevant as many NDDs show a sex bias (5). However, the mechanism(s) behind these processes are not well understood. Interestingly, work from the Rosin lab demonstrates a male-specific sensitivity of hypothalamic microglia to maternal stress, as intermittent cold exposure (i.e., 30 min. daily exposure to 4°C from embryonic day 11.5 (E11.5) to E15.5) resulted in the downregulation of 738 genes in embryonic male mouse microglia as compared to only 70 genes in females, with upregulated genes showing similar sex differences. Even more importantly, this change led to microglia-dependent social deficits in adult male offspring (5). Given the sex differences in NDDs, understanding the mechanism(s) behind these responses is critically important. Accordingly, the objective of my project is to determine how maternal cold stress exposure during pregnancy influences the microglial phenome (e.g., morphology, movement, phagocytosis), as well as to explore potential sex differences in microglial physiology under normal homeostatic conditions. ***I hypothesize that maternal cold stress exposure will alter the morphology, movement, and phagocytic activity of microglia in the fetal hypothalamus in a sex-specific manner***. This hypothesis will be tested across three aims:

Aim 1: To analyze sex differences in microglial morphology under homeostatic and stress conditions in the embryonic hypothalamus. We will begin by analyzing morphological differences in microglia from the E15.5 hypothalamus of male and female mouse embryos under homeostatic and maternal cold stress conditions. Single cells will be extracted from IBA1-stained immunofluorescent images, and a pipeline developed by the Ciernia Lab (1) will be used to generate binary images, perform skeletonization, and extract morphological features using Fiji (ImageJ). Data will then be processed in R using principal component analysis (PCA) for dimensionality reduction, followed by K-means clustering to enable unbiased classification of microglial morphologies across sex and treatment groups. We will further extend this analysis using the published MorphoGlia pipeline (3). This method employs random feature selection to identify key descriptors, followed by UMAP and HDBSCAN to characterize and cluster microglial phenotypes. Combining these two analytical approaches will provide a comprehensive assessment of microglial morphology and enable a direct comparison of their performance. This will allow us to evaluate the relative strengths and applicability of each strategy for future studies.

Aim 2: To quantify hypothalamic microglial engulfment under homeostatic and maternal stress conditions. Because 2D morphological analysis alone cannot fully capture microglial phagocytic activity, we will then assess phagocytic behavior. Using the *Cx3cr1*^{GFP} transgenic mouse line to fluorescently label microglia, we will apply pHrodo-conjugated bacterial particles to ex vivo E15.5 embryonic hypothalamic tissue. These pH-sensitive particles remain non-fluorescent extracellularly but emit red fluorescence upon internalization and acidification within phagosomes, enabling quantification of phagocytic activity. Flow cytometry (CytoFLEX) will be used to assess the proportion of phagocytic microglia and mean fluorescence intensity (MFI) across sex and treatment groups. As phagocytosis is a key function of microglia, this aim will extend the analysis and interpretability of our morphological findings.

Aim 3: To characterize hypothalamic microglial movement and activity under homeostatic and maternal stress conditions. Finally, we will evaluate whether maternal cold stress alters microglial motility and cellular interactions in the E15.5 hypothalamus of male and female embryos. Using the *Cx3cr1*^{GFP} transgenic line to label microglia, we will perform live-cell imaging via confocal microscopy to track microglial movement, speed, and contacts with neighboring cells. This region-specific analysis will be conducted in Imaris and will integrate morphological, behavioral, and phagocytic data to provide a comprehensive assessment of microglial functional states.

Significance: Collectively, findings from this research will deepen our understanding of the potential sex-specific roles microglia play during neurodevelopment and demonstrate how prenatal exposure to maternal stress impacts these processes.

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