From Nervous System Changes to Systemic Change in Science: Investigating Sex Differences in the Impact of Maternal Immune Activation on Offspring Brain Development and Behavior, and Building Social Justice in Science Through Pedagogy and Community Initiatives to Increase Belonging

By

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Abstract

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Laboratory research on sex differences in response to maternal immune activation:

We investigated sex differences in molecular and behavioral responses to maternal immune activation during pregnancy. Epidemiological evidence indicates that immune activation during pregnancy impacts brain development, leading to increased incidence of neurodevelopmental disorders such as autism spectrum disorder (ASD) and schizophrenia. Researchers have modeled this association in the laboratory using maternal immune activation (MIA) animal models, in which offspring exposed to MIA in development demonstrate changes in social and restricted repetitive behavior that recapitulate aspects of autistic behaviors in humans. My research seeks to understand the nature of and mechanisms underlying sex differences in the fetal response to maternal immune activation. Male offspring generally demonstrate greater magnitude and/or number of molecular and behavioral changes in response to immune activation during gestation compared to females. Sex differences in the MIA model are understudied, and little research has considered sex as a biological variable with the potential to impact immune response mechanisms in the MIA context.

We first used a hypothesis-driven approach to investigate the role of sex hormone pathways in macrophages as a potential sex-specific mechanism regulating the immune response in the fetal brain. The estrogen receptor β (ERβ) has been shown to negatively regulate transcription of pro-inflammatory immune response genes in microglia. We hypothesized that female and male embryos may differ in the level of ERβ signalling in brain macrophages, leading to differential immune responses and therefore the observed sex differences in neurodevelopment after maternal immune activation. To test this hypothesis, we used the Cre-Lox system to generate mouse offspring with a knockdown of estrogen receptor β (ERβ) expression in myeloid cells using cre recombinase driven by the Cx3cr1 promoter (Cre+). We then compared the behavior of Cre+ and Cre- offspring, with or without maternal immune activation via polyinosinic–polycytidylic acid (Poly(I:C)) at embryonic day 12.5. We observed the adult behavior of 94 offspring from 6

vehicle-treated and 9 Poly(I:C)-treated litters, using 11 assays measuring social, restricted repetitive, anxiety-like, depression-like, and motor behaviors. Overall we found few significant differences between experimental groups in this study, potentially due to a low 2mg/kg dose of Poly(I:C), reduced statistical power because of unexpectedly low sample sizes for control groups, and sources of variability specific to the MIA model such as maternal immune responsiveness. However, we did observe a few significant behavior differences, including: 1) baseline sex differences in marble burying with increased number of buried marbles in males, 2) female-specific effects of Cre genotype and Poly(I:C) treatment in the tail suspension test (TST), a measure of depression-like behavior, and 3) a decrease in distance traveled in the elevated plus maze in Poly(I:C)-treated animals. These findings suggest potential roles for sex in modulating restricted repetitive behavior and depression-like behavior, for macrophage ERβ signaling in regulating depression-like behavior in females, and for Poly(I:C) maternal immune activation in altering depression- and anxiety-like behavior. However, the lack of expected Poly(I:C)-induced changes in social and restricted repetitive behavior made it difficult to evaluate our hypothesis about the role of macrophage ERβ in sex differences after MIA.

We next undertook an exploratory single-cell RNA sequencing study to better understand sex differences in molecular pathways in the fetal brain in response to maternal immune activation. We stimulated pregnant female mice with 10mg/kg Poly(I:C) at E12.5 based on a dosing study that found sickness phenotypes but not litter loss at this dose. We isolated fetal brains 6 or 24 hours after injection, pooling equal numbers of fetal female and male brains at 6 hours and preparing separate female and male samples at 24 hours. We enriched for myeloid cells and prepared single cell RNA sequencing libraries using a fixation and plate-based protocol. We sequenced 975 million 150bp paired-end reads, resulting in 18465 cells and 16091 features in the final data set after filtering. Clustering resulted in 13 clusters, and cell types were identified using marker gene expression as well as comparison to reference data sets. Cell types included ependymal cells, myeloid cells, neural cells, and endothelial cells. Differential gene expression analysis between experimental groups within cell type clusters found 62 significantly differentially expressed genes (DEGs), with the majority observed in ependymal cells. Further examining the DEGs using gene set analysis, we observed 66 significant gene ontology terms for the set of 11 genes upregulated in female ependymal cells 24hr after injection with Poly(I:C) compared to vehicle, primarily relating to regulation of the immune response. We also found significant gene ontology terms relating to cilia, cell transport, and motility when examining the genes differentially expressed in male neural progenitor cells/radial glia 24hr after injection with Poly(I:C) compared to vehicle. These findings represent the first evidence to our knowledge that ependymal cells in the fetal brain may be responding to maternal immune activation. In addition, sex differences in fetal ependymal cells have not been previously reported. The observed transcriptional immune response in female ependymal cells and upregulation of cilia genes in male neural progenitor cells 24hr after Poly(I:C) treatment add to our understanding of fetal responses to MIA as well as sex differences in early brain development.

Evidence-based teaching and learning with the MCB Distance Learning Task Force:

In Spring 2020, instruction and learning at UC Berkeley largely shifted from in-person to online due to the COVID-19 pandemic. To assist this transition within the Molecular and Cell Biology

(MCB) Department and promote effective and equitable pedagogy, I founded and led the MCB Distance Learning Task Force. Collectively we designed and analyzed an undergraduate survey on the remote learning experience, and published a website for instructors with recommendations on teaching remotely based on the education literature. In our survey, administered in July 2020, 134 students reported their experience on a breadth of issues, including: 1) an overall negative impact of remote instruction on their learning, 2) their preferences for specific learning formats and technologies. 3) barriers to attending synchronous classes, 4) generally reliable access to technology and internet, 5) service needs, 6) understanding of and preferences for grading policies, and 7) concerns about cheating and equity on exams. These findings expand our understanding of MCB students' experiences and preferences about remote learning during the initial phase of the COVID-19 pandemic. Our literature review of best practices in STEM and online education was summarized in four main recommendations to instructors and published as a website with supporting information. Our main recommendations included: 1) Create an inclusive online learning environment, 2) Provide clear course materials organized within a structured website, 3) Help students connect with each other and instructors, and 4) Develop effective and fair assessments. Combining information gathered from our survey as well as our literature review, we compiled recommendations for MCB departmental leadership on actions to take to improve the remote learning and teaching experience, as well as hosted 2 training sessions for faculty and graduate student instructors on best practices. The information and recommendations the Task Force gathered apply beyond the specific context of the COVID-19 pandemic, and we hope the resources we developed will continue to be used during in-person learning and to weather future crises. Improving classroom culture, increasing student engagement, communicating about course and department policies, updating assessment structures, and providing services to students in need will always be critical to teaching and learning effectively.

Program evaluation with the Inclusive MCB initiative:

Systems of oppression within science and academia have led to discrimination of underrepresented minority (URM) and other marginalized scientists. The Inclusive MCB (iMCB) initiative was formed to create capacity within the MCB department at UC Berkeley to discuss and address issues affecting URM scientists. Among the programs initiated by iMCB include annual conferences presenting scholarly research about improving scientific culture and practices in pursuit of equity, highlighting the experiences of URM scientists, and providing opportunities to build community. The impact of the conferences were assessed by the iMCB Assessment Team using pre- and post-conference surveys. Here, I present findings from our assessment of the Fall 2020 "Building a Sense of Belonging" iMCB Conference. We found that the keynote talks and affinity groups were the most impactful components of the conference program, and that keynotes were rated more impactful by URM participants than non-URM participants. Attendees also had a stronger sense of belonging to the community of scientists and an increased comfort sharing about their values and identities after the conference. Sense of belonging increased for both URM and non-URM groups, and gaps in sense of belonging were observed both before and after the conference, with non-URMs having a higher sense of belonging in both samples. We found that respondents planned to be involved in justice, equity, diversity, and inclusion work in the coming year to a greater extent after the conference. Our results showed a clear

positive impact of the conference for both URM and non-URM participants, leading to increased personal sense of belonging as well as sentiments like comfort sharing values and plans to be involved that have the potential to impact interpersonal and organizational culture. Our results also demonstrate the continued inequities in science, with non-URM participants having a higher sense of belonging than URM participants overall. These findings underscore the need to continue improving culture and policies in science and point toward potential avenues for organizing. Indeed, these results inspired organizers to create an 8-week affinity groups program in summer 2021 and continue hosting keynotes and affinity groups at future annual conferences.

To my family - original and chosen. You made this work possible.

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Preface

The work described in this dissertation includes both laboratory research and program evaluation for social justice organizing. Though these topics may seem distinct to some, my work in both areas has been inextricably linked and these parallel efforts have challenged and enriched one another. I have included pedagogy and community organizing work in my dissertation because it is important to me both professionally and personally. At the professional level, I believe that as a scientist I am obligated to work towards improving the culture and policies of the scientific enterprise until it is equitable and accessible to all scientists, and our research priorities reflect the needs of society more broadly. By including program evaluation chapters in my thesis, my goal is to situate pedogogical reform and social justice work as equally important components of what it means to "do science."

On a personal level, the social justice work I did during my PhD enabled me to persist in my laboratory work because I had the opportunity to positively impact others, work toward social change, and build meaningful community through teaching and organizing. Like many other women and under-represented minority scientists, I experienced "communal goal incongruity" during my research career (Boucher et al., 2017). I value helping others and bettering society, but this goal isn't always valued or a top priority in scientific contexts. Finding communities of scientists who also valued social justice was critical to my sense of belonging to the broader scientific community, knowing I could be both things, an activist and a scientist. There is no doubt in my mind that the scientific work I detail here would not exist without the accompanying social justice work. Unfortunately, the positive impact this work had on me and my scientific career is not always the case for organizers, especially people with marginalized identities that get targeted for their involvement or worn down by the roadblocks to change. I am grateful for the incredible communities and mentors I found in my cohort, lab, iMCB initiative, faculty, and staff at Berkeley. These people supported my interest, commitment, and growth in practicing science responsibly and building better communities in science.

Part I

Sex Differences in the Brain and Behavior in Response to Immune Activation During Fetal Development

1 Introduction to the Immune System and Sex as Variables in Brain Development

My research investigates sex differences in fetal brain development and adult behavior in the context of immune activation during pregnancy. I study these processes in mice using the maternal immune activation (MIA) animal model, which has been proposed to model some of the molecular and behavioral aspects of autism spectrum disorder (ASD). MIA and ASD are biologically complex - they sit at a crossroads between multiple organ systems and developmental processes, including the immune system, brain development, and sexual differentiation. Zooming out, they encompass interactions between genetic and environmental variables, and between fetal and maternal bodies. In the broadest view, studying ASD biology requires taking perspectives from the scientific to the sociological. The following chapter seeks to introduce the reader to the main systems and variables important for understanding the primary research in subsequent chapters.

A brief note on terminology: I will be using primarily "ASD", "on the autism spectrum", or "autistic" to refer to autism, autistic people, and autistic traits. I have sought to avoid ableist language to respect the desires and humanity of the autistic community, recognizing that individuals on the autism spectrum may have specific language preferences that are not reflected here (Bottema-Beutel et al., 2021; Keating et al., 2022).

1.1 Immune System in Brain Development and NDDs

The immune system during pregnancy has long been viewed as a conflict between fetal antigens and maternal immunity, with the idea that immuno-suppression allows for pregnancy to proceed (Medawar and Medawar, 1953; Rendell et al., 2020). The truth is likely more complicated. The maternal immune system dynamically adapts to the phase of pregnancy, and maternal, placental, and fetal immune systems work together to maintain homeostasis and respond to pathogens (Mor and Cardenas, 2010; Arck and Hecher, 2013; Abu-Raya et al., 2020). Early in pregnancy, maternal immune cells including NK cells and dendritic cells are required for proper implantation of the blastocyst and creation of the uterine decidua (Hanna et al., 2006; Plaks et al., 2008). As pregnancy progresses, the developing placenta becomes an immune organ regulating maternal immune processes and protecting against pathogens at the maternal-fetal interface (Ding et al., 2022). Ultimately, the fetus develops its own functioning immune system that can play a role in

responding to environmental and maternally-based cues (Rechavi and Somech, 2019; Hossain et al., 2022).

Finely tuned regulation of this complex maternal-fetal immune system is critical for successful development of pregnancy for both the parent and offspring (Mor and Cardenas, 2010). Pregnancy outcomes and maternal health can be adversely affected by dysregulation or activation of the maternal or placental immune systems. For example, systemic inflammation, placental inflammation, activation of the complement system, and viral infections have been associated with increased risk of outcomes such as preeclampsia, early pregnancy loss, preterm birth, and low birth weight (Gichangi et al., 1993; Kim et al., 2015; Regal et al., 2015; Pfeifer and Bunders, 2016; Han et al., 2019; Aneman et al., 2020; Chudnovets et al., 2020; Epelboin et al., 2021). In addition to the impact on birth outcomes, inflammation and immune cell activation have been linked to prenatal fatigue and postpartum depression (Guintivano et al., 2022; Xia et al., 2022). Further suggesting immune heterogeneity and complexity in pregnancy, autoimmune diseases can go into remission *or* be exacerbated during pregnancy (Munoz-Suano et al., 2011).

Immune activation not only impacts the parental body and overall fetal development outcomes, but also particularly impacts specific fetal systems. The fetal brain is a highly studied site where inflammation during pregnancy influences development and long term outcomes (Rees and Inder, 2005; Knuesel et al., 2014). Congenital neurological abnormalities are well-known symptoms of *in utero* infections with so-called TORCH pathogens (*Toxoplasma gondii*, rubella, cytomegalovirus, and herpes simplex virus, and others) and more recently Zika virus (Lu-Culligan and Iwasaki, 2020).

Neuropsychiatric conditions with developmental origins, such as autism spectrum disorder (ASD) and schizophrenia, have also been linked to infection during fetal development (al-Haddad et al., 2019). A rubella outbreak in 1964 led to a series of reports of several-fold higher ASD prevalence in children whose mothers were infected with rubella during pregnancy (Chess, 1971). Additional reports over the years have found higher rates of ASD for other types of infections, including when mothers had a fever, bacterial infection, influenza, multiple infections over pregnancy, or required hospitalization (Atladóttir et al., 2010; Atladóttir et al., 2012; Zerbo et al., 2013; Lee et al., 2015; Jiang et al., 2016). One report investigating influenza infection during pregnancy and ASD diagnoses in children found no association, however (Zerbo et al., 2017). In addition to these ASD studies, a large body of work has associated schizophrenia with several types of viral infection during pregnancy, including influenza (Brown and Derkits, 2010).

Clinical studies have also observed biomarkers of immune dysregulation during pregnancy for mothers of children on the autism spectrum. A Finnish national birth cohort study found a positive association between ASD and C-reactive protein levels in maternal serum (Brown et al., 2013). Jones et al. (2016) found elevated interferon-gamma, Interleukin-1 alpha**,** and interleukin-6 (IL-6) in maternal serum for ASD cases with intellectual disability (ID), and reduced interleukin-8 for ASD cases without ID. Additionally, maternal antibodies recognizing fetal brain proteins have been implicated in the etiology of ASD, though how this pathway may be related to other immune-mediated mechanisms contributing to ASD, such as infection, is currently unclear (Braunschweig and Van de Water, 2012). Immune stressors during pregnancy beyond or in addition to pathogenic

infection may also play a role in neurodevelopmental disorders (NDDs). Environmental factors may modulate or interact with immune factors to alter neurodevelopment, including air pollution and food insecurity (Bilbo et al., 2018; Volk et al., 2020; Mate et al., 2021).

1.2 Behavior and Immune Phenotypes in ASD

Autism spectrum disorder (ASD) is a common neurodevelopmental disorder with an estimated worldwide prevalence of 1% (Zeidan et al., 2022). ASD is defined diagnostically by two behavioral domains: 1) deficits in social communication and interaction, and 2) restricted, repetitive behavior or interests (American Psychiatric Association, 2022). Autism co-occurs with other neurodevelopmental and psychiatric conditions including ADHD, anxiety and mood disorders, and obsessive compulsive disorder, with >70% of people on the autism spectrum experiencing at least one co-occurring condition (Vohra et al., 2016; Rosen et al., 2018).

Immune dysregulation has emerged as an important co-occurring pattern in people on the autism spectrum, beyond the epidemiological link to maternal infection. Genetic association studies have identified immune signaling (along with synaptic transmission and transcriptional regulation) as a key process associated with ASD (De Rubeis et al., 2014; Krishnan et al., 2016). Cytokine and transcriptome analyses in autistic people have highlighted immune dysregulation as a feature of autism; reports have measured elevated cytokines in plasma and cerebrospinal fluid, as well as upregulation of immune-related transcripts and activation of glia in brains from people on the autism spectrum (Vargas et al., 2004; Chez et al., 2007; Garbett et al., 2008; Ashwood et al., 2011).

1.3 Sex Differences in ASD

Many neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), and schizophrenia, show sex differences; yet, the biological mechanisms behind these observations are poorly understood (Waddell and McCarthy, 2010; Hanamsagar and Bilbo, 2016; Hill, 2016; McCarthy, 2016; Bordeleau et al., 2019; May et al., 2019; Lord et al., 2020; Merikangas and Almasy, 2020). For example, it is known that males are diagnosed with ASD at higher rates than females (Baron-Cohen et al., 2011; Loomes et al., 2017; Dietz et al., 2020). A meta-analysis indicates a male to female ratio of approximately 3:1 or 4:1 in ASD (Loomes et al., 2017).

One contributing factor to this sex difference in incidence is social in origin: diagnosis rates. Ratios are typically closer to 3:1 for screening studies compared to higher estimates when examining people who already have an ASD diagnosis, likely do to social factors leading to reduced rates of diagnosis in females (Russell et al., 2010). Autistic traits vary by sex, with females more likely to have co-occurring internalized disorders and to exhibit camouflaging, which may also contribute to differences in diagnosis rates (Hull et al., 2020). As more autistic women and non-binary

people speak out about the gendered nature of diagnosis and the need for reforms, diagnostic rates may be less male-biased (Astra, 2022). However, sex differences in incidence and autistic trait presentation are unlikely to disappear even with improved diagnosis, and studying the biology underlying these differences may allow for better interventions for autistic individuals of all sexes.

Several theoretical frameworks have been proposed to explain the sex difference in ASD incidence. In general terms, the presence of either a female-specific protective effect, a male-specific risk, or both, will cause a population-wide shift in susceptibility relative to the other sex (Werling and Geschwind, 2013). Supporting a general female protective effect, twin studies and genetic data have shown an increased "etiological load" in females compared to males on the autism spectrum (Robinson et al., 2013; Jacquemont et al., 2014). One possible source of sex differences in ASD etiology are sex chromosome gene effects. Indeed, mutations in many genes are known to increase the probability of ASD, and some of them, such as *FMR1* (fragile X messenger ribonucleoprotein), *MeCP2* (methyl-CpG binding protein 2), *NLGN3,* and *NLGN4* (neuroligins 3 and 4), are on the X-chromosome (Marco and Skuse, 2006; Guy et al., 2011; Percy, 2011; Zhang et al., 2016; Sledziowska et al., 2020; Savatt and Myers, 2021). While it will not be addressed here, excellent reviews that discuss the chromosomal contributions to sex differences in ASD can be found elsewhere (Marco and Skuse, 2006; Ferri et al., 2018; Green et al., 2018; Savatt and Myers, 2021). Fetal testosterone (fT) has been proposed as a male-specific molecular risk factor, and several studies have associated fT or elevation of multiple fetal steroids with autistic-like traits, however, the underlying mechanism linking fT and ASD has not been elucidated (Baron-Cohen et al., 2011; Baron-Cohen et al., 2015). Further implicating sex hormones, endocrine disruption during fetal brain development increases the risk of NDDs (Colborn, 2004; Schug et al., 2015; Moosa et al., 2018).

Differential susceptibility to neuroinflammation in males and females has also been proposed as a source of sex differences in ASD (Hanamsagar and Bilbo, 2016). In the section below, "Role of estrogen receptor beta in regulation of immune responses," I will discuss the hypothesis that regulation of inflammation by sex hormone nuclear receptors (NRs) contributes to the observed sex differences in ASD.

1.4 Sex Differences in Immunity

Though both sex differences and immune involvement are well established features of ASD, mechanisms linking sex and immune factors in neurodevelopmental disorders like ASD are not as well studied. However, the importance of sex in inflammation has been demonstrated in other biological contexts. Sex-dependent inflammatory phenotypes are observed in response to innate and adaptive immune reactions as well as in acute and chronic inflammatory diseases and their animal models (Klein and Flanagan, 2016; Chamekh and Casimir, 2019; Gal-Oz et al., 2019). Males are generally more susceptible to pathogen infections, while females are more often diagnosed with autoimmune diseases (Klein, 2012; Quintero et al., 2012; Ngo et al., 2014; Vázquez-Martínez

et al., 2018; Billi et al., 2019; Lasrado et al., 2020) . For example, in experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, female and male mice have differing disease courses (Constantinescu et al., 2011). Phenotypes also differ by sex in animal models of high-fat diet, which induces low grade but chronic inflammation in macrophages and disrupts homeostasis in adipose tissues, resulting in induction of metabolic syndrome (Lumeng et al., 2007; Duan et al., 2018). Male mice gain weight and display insulin resistance, while female mice are more resistant to these effects (Pettersson et al., 2012; Casimiro et al., 2021). These observations suggest that sex-specific factors are important in regulating inflammation.

A few research groups have sought to link immune processes and the sex differences observed in NDDs. A longitudinal case control study investigating associations between schizophrenia and prenatal cytokines found sex dependent effects, with schizophrenia-associated elevations in IL-6 in males, and reductions in Tumor necrosis factor a in females (Goldstein et al., 2014). Another study investigating maternal cytokines stratified by trimester found that specific combinations of cytokines at specific times were associated with sets of psychiatric symptoms, with higher interleukin 1 receptor antagonist in the second trimester being associated with increased internalizing symptoms only in females (Mac Giollabhui et al., 2019). Transcriptomic analysis of brain samples from people on the autism spectrum found male-biased expression in glial/immune gene modules and female-biased expression in neuronal/synaptic modules, suggesting sex-dependent etiologies and greater immune involvement in autistic males (Kissel and Werling, 2022). Though human studies examining the interaction of sex and immunity in NDDs are rare, pre-clinical animal models of maternal immune activation have successfully recapitulated 1) sex-specific features 2) immune involvement 3) and behavior changes relevant to NDDs. This model and associated sex differences are described below.

1.5 Maternal Immune Activation Model of NDDs and Observed Sex Differences

To replicate the immune activation component of ASD in animal models, researchers developed the Maternal Immune Activation (MIA) paradigm. Pregnant rodents or primates are injected with virus or toll-like receptor agonists to stimulate antibacterial or antiviral responses (Shi et al., 2003; Estes and McAllister, 2016; Lu-Culligan and Iwasaki, 2020). A commonly used ligand is polyinosinic:polycytidylic acid (Poly(I:C)), which mimics infection by double-stranded RNA viruses and triggers the TLR3-mediated innate immune response (Smith et al., 2007; Patterson, 2011). Induction of MIA can induce behaviors in the adult offspring reminiscent of ASD phenotypes. Behavioral phenotypes in MIA offspring include decreased sociability, increased repetitive restricted behavior, impaired learning and memory, altered levels of anxiety, and hyperactivity (Patterson, 2011; Estes and McAllister, 2016). Based on these findings, MIA induction in mice is widely used to study the mechanism of ASD because it successfully recaptures behavioral phenotypes via immune pathways, a mechanism known to be involved in ASD.

Several pathways have been implicated in the generation of neurological phenotypes in MIA offspring. Pioneering work from the Patterson lab revealed the importance of the cytokine IL-6 in mediating the effects of Poly(I:C) on offspring (Smith et al., 2007). The Choi and Huh labs demonstrated that maternal Th17 T cells and the cytokine interleukin-17a were required for MIA-induced phenotypes, found alterations in layering in a region of the somatosensory cortex, and observed male-specific activation of the integrated stress response in neural cells (Choi et al., 2016; Kalish et al., 2021). Placental damage and fetal hypoxia have also been proposed as mechanisms for propogation of MIA signals to the fetal brain, resulting in sex-specific effects like decreased neural progenitor cell proliferation in the cortex of male offspring and reduced body growth in females exposed to lipopolysaccharide (LPS) during development (Carpentier et al., 2013).

MIA models appear to recapitulate basic sex differences observed in ASD. Some MIA researchers, including our lab, have reported sex-specific molecular or behavioral phenotypes in MIA offspring (Xuan and Hampson, 2014; Coiro and Pollak, 2019; Haida et al., 2019; Keever et al., 2020; Nichols et al., 2020; Kalish et al., 2021). Unfortunately, sex differences in the model have not been consistently studied or reported (Coiro and Pollak, 2019). Studies may only measure males or fail to disagreggate by sex, and some of the most cited reviews of the impact of maternal immune activation on the brain and the associated MIA animal models barely mention or don't mention sex at all (Knuesel et al., 2014; Estes and McAllister, 2016). This lack of research including sex as a biological variable is an issue across biology and neuroscience, not necessarily specific to the MIA field, though many ASD-related studies have justified studying only males due to the male-biased diagnostic ratio (Beery and Zucker, 2011; Geller et al., 2018; Sohn, 2019; Arnegard et al., 2020).

Of the studies examining molecular mechanisms of MIA that report results by sex, data generally point to a greater degree of MIA-induced changes in males compared to females. Kalish et al. (2021) reported male-specific reduction in protein translation and activation of the immune response after Poly(I:C) MIA at embryonic day (E) 12.5, though they did note a few female-specific proteins with increased translation after MIA. A study investigating microglial morphology in adult brains found male-specific changes in morphology and arborization after MIA (Hui et al., 2018). In a porcine model of MIA, microglia-enriched genes were differentially expressed 21 days after viral inoculation, with decreased expression in females and increased in males after infection (Antonson et al., 2019). Interestingly, due to baseline sex differences in expression, MIA resulted in males and females having more similar expression of these genes. This study suggests that immune activation may alter innate sexual differentiation processes in microglia.

In MIA behavior studies reporting on sex differences, results are variable. In some studies, only males display phenotypic changes (Nichols et al., 2020; Vitor-Vieira et al., 2021). Several studies have observed task-dependent sex-differences in behavior, including some impacts of MIA on female behavior (Hui et al., 2018; Braun et al., 2019; Carlezon et al., 2019; Gogos et al., 2020). For example, Ingrid C Y Xuan and David R Hampson (2014) found that both female and male mice had changes in social behavior after Poly(I:C), but only males showed increased marble burying (a measure of restricted repetitive behavior). Another study from mice exposed to Poly(I:C) at E9.5 observed increased marble burying and decreased sociability in both females and males, but increased anxiety-like behavior only in males (Hui et al., 2018). This variability in reported sex

differences is likely a product of differences in immune stimuli and developmental timing between laboratories, as well as the inherent variability in the model that has been recently detailed in excellent reviews (Kentner et al., 2018; Bauman and Van de Water, 2020). Though the variability can hinder reproducibility and potentially require larger cohorts, it can also be a potential avenue for uncovering the factors that make NDDs heterogeneous in penetrance and presentation.

1.6 Role of Brain Myeloid Cells in Brain Development

Microglia and other brain macrophages have been proposed as critical fetal cell types that may respond to maternal immune activation and mediate its impact on fetal brain development (Prins et al., 2018; Smolders et al., 2018; Otero and Antonson, 2022). Brain myeloid-lineage cells derive from primitive macrophages in the yolk sac and migrate to the brain on E9.5 in mice, after which these cells expand, migrate, and develop into microglial cells and border-associated macrophages (BAM) (Ginhoux et al., 2010; Goldmann et al., 2016; Utz et al., 2020). These two major myeloid subsets have common as well as subset-specific gene expression profiles and localize to different areas of the brain: microglia in the brain parenchyma, and BAM in perivascular areas, the meninges, and the choroid plexus (Ginhoux et al., 2010; Goldmann et al., 2016; Mrdjen et al., 2018; Jordão et al., 2019; Van Hove et al., 2019; Utz et al., 2020). Microglia, the resident immune cells in the brain, have immune properties such as competence to sense pathogens, phagocytose, and secrete cytokines (Kettenmann et al., 2011). In addition to their immune functions, microglia have important roles in brain development, including regulating neurogenesis and refining synapses (Ginhoux et al., 2010; Tremblay et al., 2010; Cunningham et al., 2013). Indeed, studies depleting microglia or inducing microglial activation demonstrate their importance in regulating proper development of neural circuits (Pont-Lezica et al., 2014; Squarzoni et al., 2014; Oosterhof et al., 2019).

1.7 Evidence for Brain Myeloid Cell Involvement in Maternal Immune Activation

Though studies investigating changes in microglia density and morphology after MIA have reported conflicting results, recent transcriptome analyses of isolated microglia indicate that MIA induces increased expression of immune pathways and decreased expression of genes involved in nervous system development (Pont-Lezica et al., 2014; Smolders et al., 2018). A few studies point to BAM as a key cell type in the response to MIA in the fetal brain. Although the precise mechanism is not clear, a recent publication indicates that MIA-activated BAM in the choroid plexus secrete the chemokine CCL2 into the fetal ventricle, resulting in enhanced local inflammation (Cui et al., 2020). Moreover, a single-cell RNA-sequencing (scRNA-seq) study from our lab showed that the activation of fetal BAM in response to MIA was dependent upon fetal *Trif*, an essential signaling molecule downstream of TLR3 (Nichols et al., 2020). These findings indicate that MIA leads to fetal innate immune signaling in BAM. Furthermore, in validating this scRNA-seq

data, we found that MIA causes BAM in the choroid plexus, but not meningeal BAM or microglia, to have increased expression of *S100a8 and 9*, key inflammatory genes that are known to induce chemotaxis and enhance inflammation (Ehrchen et al., 2009; Cesaro et al., 2012; Cury et al., 2013; García-Arias et al., 2013; Sherwood and Walsham, 2016; Nishikawa et al., 2017; Aranda et al., 2018; Wang et al., 2019; Silvin et al., 2020). Alteration of gene expression programs in fetal microglia or BAM cells after E12.5 PolyI:C stimulation could prevent them from performing their normal developmental functions, resulting in aberrant brain development.

1.8 Role of Estrogen Receptor Beta in Regulation of Myeloid Immune Responses in the Brain

Since MIA induces inflammation in brain myeloid-lineage cells, one hypothesis to explain the male bias in ASD is differing magnitude and duration of inflammation in males and females during fetal development. In this section, I will discuss sex steroid nuclear receptors, especially estrogen receptor (ER) β, as potential regulators of fetal brain inflammation. I focus on ERβ because (1) ERβ is broadly expressed in mouse brain and (2) our lab previously showed that ERβ could regulate inflammation in microglial cells (Mitra et al., 2003; Fan et al., 2006; Saijo et al., 2011).

1.8.1 Expression of Estrogen Receptors and Sex Steroid Hormones in the Fetal Brain

So far, it is not clear whether ERα and ERβ expression in the myeloid cells of the fetal brain varies by sex. Studies have examined estrogen signaling primarily in whole brain or neuronal cells, and few have examined developmental time points prior to the neonatal period. Excellent reviews are available for overall brain expression analyses of ERα, ERβ, and enzymes required for the generation of androgens and estrogens (McCarthy, 2008; Bondesson et al., 2015). Several reports indicate that ERα, ERβ, and enzymes that metabolize sex hormones are present during mid-gestation. For example, ERβ expression was detected in the fetal midbrain, neuromere, hypothalamus, thalamus, and basal plate of pons at E12.5, and ERα expression was observed at E16.5 in a gonadal sex dependent manner (Fan et al., 2006; Cisternas et al., 2015). In amygdala neuronal cultures obtained from E15 embryos, ERβ is sex-differentially regulated: lower levels of *Esr2* mRNA expression were observed in females, but also sex differences in hormonal responsiveness were present, with increased *Esr2* expression in response to 17β-estradiol or dihydrotestosterone (DHT) hormonal stimulation only in females. These effects were dependent on sex chromosome complement (Cisternas et al., 2017). Activity of ERs, using an ERE-luciferase reporter, was observed in the fetal forebrain and hindbrain as early as E13.5, though no difference was detected between brains from females and males except in the P1 hindbrain (Della Torre et al., 2018). Several key proteins involved in steroid hormone synthesis are expressed in female and male E16 fetal brain, including the transport protein steroidogenic acute regulatory protein, and the enzymes cytochrome P450 family 11 subfamily a member 1, 5α-Reductase, and aromatase (Cisternas et al.,

2015). Aromatase is an enzyme that converts testosterone to 17β-estradiol and androstenedione to estrone. Notably, sex-dependent expression of aromatase in the developing mouse brain has been reported, which may indicate the presence of differing concentrations of ER ligands in females and males that could impact downstream signaling (Harada and Yamada, 1992; Greco and Payne, 1994; Hutchison et al., 1997; Cisternas et al., 2015; Shay et al., 2018; Sellers et al., 2020).

Little is known about the expression of sex steroid hormones in the fetal mouse brain; however, a report showed that 17β-estradiol, testosterone, and DHT were detected in the brains of fetal mice, and that these hormones may exhibit sex dimorphic expression patterns in different brain regions (Konkle and McCarthy, 2011). However, to better understand how sex steroid hormones may regulate inflammation induced by MIA, precise analysis of sex steroid hormone expression in the fetal brain will be important.

Together, these expression studies suggest that the cellular machinery for ER signaling is present in the fetal brain from a relatively early age, and that sex differences in the expression of receptors, steroid metabolizing enzymes, and hormone ligands could contribute to differential regulation by ERs in females and males. Our preferred hypothesis is that concentrations of particular ER ligands differ between females and males in such a way that MIA-induced inflammatory responses differ in magnitude or duration. For example, ligands that induce transcriptional repression of inflammatory genes via ERβ may be highly expressed in female fetal brains, leading to efficient resolution of inflammation upon MIA. The hypothetically lower expression of such repressive ERβ ligands in fetal male brains could result in larger or prolonged inflammatory responses compared to females. A comprehensive analysis of the expression of ERs and related ligands in developing fetal mouse brains, especially comparing sex, cell type, and specific brain region, will be important in understanding the contribution of ER-mediated transcription in sex-specific brain development.

1.8.2 Nuclear Receptor Signaling in General

NRs are a family of transcription factors which both positively and negatively regulate transcription in response to ligand binding or other mechanisms. Steroid hormone NRs are a class of NRs with activities that depend on endogenous small lipophilic ligands such as steroid hormones. For example, estrogen receptors (ERs) bind to estrogen response elements (essential ERE, 5'-GGTCAnnnTGACC-3') in gene regulatory regions to control the expression of target genes (Driscoll et al., 1998; Klinge, 2001). In addition to direct DNA binding, NRs can also regulate transcription by binding to other transcription factors *in trans*. NR function depends upon the ligands that are bound to the receptor. Indeed, NRs change their conformation in response to ligand binding in order to recruit either transcriptional activator or repressor complexes, and it has been proposed that ligand binding may induce post-translational changes on NRs that stabilize co-factor binding (Moras and Gronemeyer, 1998; Hammer et al., 1999; Bourguet et al., 2000; Lannigan, 2003; Nagy, 2004; Pascual et al., 2005; Lalevée et al., 2010; Anbalagan et al., 2012; Helzer et al., 2015; El Hokayem et al., 2017). To carry out their transcriptional activation and repression activities, NRs recruit a wide variety of co-factors and enzymes required for modifying histones and

remodeling chromatin. These factors include histone acetyltransferases, deacetylases, methyltransferases, demethylases, and chromatin remolding factors, as well as kinases, phosphatases, and ubiquitin and SUMO E3 ligases (Olefsky, 2001; Perissi and Rosenfeld, 2005; Dasgupta et al., 2014).

1.8.3 Estrogen Receptors and Their Impact on Inflammation

Various reports have suggested that sex steroid hormones and their steroid hormone nuclear receptors (NRs) may regulate inflammatory responses in innate immune cells. In particular, two estrogen receptor isoforms (ERα and ERβ) as well as the androgen receptor (AR) are well characterized sex steroid hormone NRs that are known to regulate innate immune responses (Vegeto et al., 2003; Baker et al., 2004; Suuronen et al., 2005; Harkonen and Vaananen, 2006; Sierra et al., 2008; Lai et al., 2009; Saijo and Glass, 2011; Kovats, 2015; Villa et al., 2015, 2016; Ardalan et al., 2019; Becerra-Diaz et al., 2020). Our lab has previously reported that ERβ regulates the duration and magnitude of the inflammatory response in microglial cells (Saijo and Glass, 2011). ERβ binds a range of ligands, including estrogens and androgens, and specific ERβ ligands can facilitate repression of inflammation (Kuiper et al., 1997; Wu et al., 2013). Several reports have indicated that 17β-estradiol, a ligand for both ERα and ERβ, can regulate inflammation in myeloid-lineage cells. However, this regulation is not always clear in that some reports have suggested that ER-mediated transcription represses inflammation, while others have suggested that it does not (Vegeto et al., 2003; Calippe et al., 2010; Ribas et al., 2011; Shindo et al., 2020) . While the amino acid sequences of the DNA-binding domains of these two ER isoforms are highly conserved, their ligand-binding domains (LBDs) are much less so (47% in human). Since the functions of NRs are dependent upon ligands, this lack of conservation in ER LBDs may suggest that ERα and ERβ may differ in their preferential ligands, and that binding of the same ligand to either ERα or ERβ could result in different transcriptional outputs.

Previously, our lab reported that ERβ represses inflammation in microglia in a ligand-dependent manner (Saijo and Glass, 2011). In mouse microglial cells, a subset of ligands, including the endogenous ligand 5-androsten-3β, 17β-diol (Δ5-Adiol) and the synthetic ligands Indazole-estrogen-Cl and -Br, have been shown to induce transcriptional repression of inflammation in an ERβ-dependent manner. Treatment with these repressive ligands, but not the classic ER ligand 17β-estradiol, results in the recruitment of the transcriptional corepressor CtBP (Saijo and Glass, 2011). CtBP is a co-repressor platform that is known to assemble enzymes required for transcriptional repression, such as euchromatic histone-lysine N-methyltransferase 2 (EHMT2, also known as G9a), euchromatic histone-lysine N-methyltransferase 1 (EHMT1, also known as GLP), the histone deacetylases HDAC1 and 2, and lysine demethylase 1A (KDM1a, also known as LSD1) (Chinnadurai, 2002; Dcona et al., 2017). When microglial cells are stimulated with the TLR4 ligand lipopolysaccharide (LPS), ERβ binds to cFos and repressive ligands, which results in the recruitment of the CtBP complex to target genes, thus regulating inflammation through a transrepression mechanism. Interestingly, mutations in ERβ, CtBP1/2, and HDACs have been observed in human ASD patients (Chakrabarti et al., 2009; Zettergren et al., 2013; De Rubeis et al., 2014). Although these NRs and their co-factors/binding partners are proposed to be genetic factors for

ASD, we consider the possibility that these steroid hormone NRs and their ligands may exert their effects on brain development by modulating the inflammatory response to environmental immune stimuli.

1.9 Summary

Immune activation during pregnancy is associated with the development of neurodevelopmental disorders such as autism spectrum disorder (ASD). To better understand the link between immune activation and brain development, researchers have used the maternal immune activation (MIA) animal model to study maternal and fetal responses to immune stimuli, as well as resulting changes in offspring behavior.

Sex differences are present in ASD, which is more common in males and has a different presentation depending on sex. Animal models of maternal immune activation have also shown some sex differences, though research is sparse and mechanisms for sex differences are unclear.

As the resident immune cells in the brain with known roles in brain development, brain myeloid cells such as microglia and border-associated macrophages are promising candidate cell types in mediating the effects of maternal immune activation in the fetal brain. Immune processes in these cells can be regulated by sex steroid hormone pathways, which could be a source of sex differences in response to MIA.

In order to better understand the mechanisms by which maternal immune activation impacts brain development, and investigate sex differences in this process, I pursued two main lines of research:

1. In a hypothesis-driven study, I used *ERβ*$^{fl/fl}$*;Cx3cr1-cre* mice to ask whether *ERβ* in brain myeloid cells regulates the development of offspring behavior changes in response to MIA in a sex-specific manner. Results from this study were inconclusive, since the expected behavior changes in social and repetitive, restricted behavior in the MIA group were not observed.
2. In a more exploratory study, I used single-cell RNA sequencing to examine the transcriptomic response in myeloid-enriched fetal brain cells 6 or 24hr after MIA, asking whether myeloid cells were acutely responding and whether sex differences could be detected in the fetal brain at this early time point. Interestingly, myeloid cells were not the main responder population in our sample; instead we found increased expression of immune response genes in female ependymal cells, the cells which line the brain ventricles, as well as expression changes in male neural progenitor cells.

The following chapters will detail these experiments, discussing the results, caveats, implications of the findings, and potential avenues for future work.

2 Investigating a sex hormone hypothesis for sex differences in behavior after maternal immune activation: role of ERβ in myeloid cells

2.1 Introduction

Autism spectrum disorder (ASD) has sex differences in prevalence and presentation, with an estimated 4:1 ratio of male to female diagnoses (Zeidan et al., 2022). Though the origins of these sex differences are unknown, sex hormones have been proposed as a modulatory factor, with research linking fetal steroidogenic activity to ASD diagnoses and sex hormone metabolism genes to ASD traits (Chakrabarti et al., 2009; Baron-Cohen et al., 2020). Maternal immune activation (MIA) has been described as an environmental variable impacting neurodevelopment and contributing to the development of ASD (Patterson, 2011; Atladóttir et al., 2012). Sex differences in behavior have also been demonstrated in MIA animal models (Xuan and Hampson, 2014; Coiro and Pollak, 2019; Haida et al., 2019; Keever et al., 2020; Nichols et al., 2020). However, research examining the interaction between immune activation during pregnancy and sex-specific mechanisms in ASD etiology has not been done.

Several lines of evidence suggest that sex hormone pathways are involved in ASD mechanisms. Altered expression of steroid biosynthesis enzymes have been associated with ASD, including elevated *SRD5A1* mRNA in lymphoblasts from autistic/control sibling pairs and decreased aromatase in post mortem cortex from autistic patients (Hu et al., 2009; Sarachana et al., 2011). Additionally, single nucleotide polymorphisms in the estrogen receptor beta gene *ESR2*, as well as steroid synthesis genes *CYP11B1, CYP17A1,* and *CYP19A1* were associated with both ASD diagnosis and autism behavioral traits (Chakrabarti et al., 2009). Sex hormones themselves have also been linked to ASD. In a Danish birth cohort, amniotic fluid samples showed increased levels of estrogens (estradiol, estrone, and estriol), androgens (adrostenedione and testosterone), and progestagens (progesterone and 17α-hydroxy-progesterone) in the group later diagnosed with ASD or pervasive developmental disorder not otherwise specified (Baron-Cohen et al., 2015; Baron-Cohen et al., 2020). Inherent differences in sex hormone pathways between females and males or sex-differential modulation of these pathways by genetic or environmental exposures, could contribute to the sex differences observed in ASD and MIA models.

In this study, we investigated steroid hormone pathways as a potential sex-specific mechanism involved in MIA phenotypes. We propose that sex steroid nuclear receptors may regulate the inflammatory response in fetal brain myeloid cells. If this regulation of inflammation occurs in a sex-specific manner, it could lead to the observed sex differences in MIA offspring behavior.

Nuclear receptors regulate transcription in a ligand-dependent manner by translocating into the nucleus and binding directly or via coactivators to DNA sequences of target genes (Novac and Heinzel, 2004). The estrogen receptor beta (ERβ) has been shown to negatively regulate transcription of pro-inflammatory genes in response to LPS stimulation in microglia (Saijo et al., 2011). Interestingly, this negative regulation only occurred when ERβ was bound to particular ligands such as the androgen 5-androsten-3β,17β-diol (Δ5-ADIOL), and excluding the classic estrogen E2 (Saijo et al., 2011). We propose that ERβ is similarly regulating inflammatory gene expression in brain myeloid cells during embryonic development, and that sex-specific hormone levels lead to sex differences in ERβ-mediated gene regulation upon prenatal Poly(I:C) stimulation. We hypothesize that the female fetal brain has a higher baseline level of the anti-inflammatory ERβ ligand Δ5-ADIOL, resulting in female protection against PolyI:C-induced inflammatory responses by brain myeloid cells such as microglia. Given the importance of microglia for brain development, more pronounced alteration of microglial biology in males could lead to male susceptibility to neurodevelopmental alterations and the observed sex differences in PolyI:C-induced behavior changes.

To test this hypothesis, we used the Cre-Lox system to selectively knock down ERβ expression in myeloid cells. We took advantage of a BAC transgenic mouse line expressing Cre recombinase under the *Cx3cr1* promoter (*Cx3cr1-cre)* and an *Esr2* exon 3 floxed mouse line ($ER\beta^{fl/fl}$) to target ablation of ERβ to cells from the myeloid lineage (Binder et al., 2013 ; Yona et al., 2013). We bred $ER\beta^{fl/fl}$ dams with $ER\beta^{fl/fl}$*;Cx3cr1-cre* sires and induced Poly(I:C) MIA in the dams, thereby generating MIA offspring litters with both $ER\beta^{fl/fl}$*;Cx3cr1-cre* and $ER\beta^{fl/fl}$ control animals. We used behavior testing to evaluate the response to MIA in the offspring. Behavior is the gold-standard outcome measure for MIA since clear and consistent molecular biomarkers for both ASD and MIA offspring have not been developed.

We measured mouse phenotypes that resemble the core features of autism - social interaction and restricted, repetitive behavior. We also examined mouse behaviors related to psychiatric symptoms that co-occur with ASD. Anxiety and mood disorders frequently co-occur in people on the autism spectrum, and several MIA animal studies observe anxiety- or depression-like behavior in adult offspring across MIA immunogen types (Meyer et al., 2007; Khan et al., 2014; Depino, 2015). Given the higher prevalence of anxiety and mood disorders in women, we wondered whether MIA-induced anxiety-like or depression-like behaviors in mice could demonstrate sex differences (sex differences were not examined in prior studies of anxiety- and depression-like behavior in MIA offspring). Furthermore, we hypothesized that regulation of the immune response in myeloid cells by ERβ could impact these co-occurring behaviors in a sex-specific manner.

2.2 Methods

2.2.1 Animals

All animal housing and experiments were approved by the University of California, Berkeley Animal Care and Use Committee.

2.2.1.1 Housing and Animal Husbandry

Mice were housed with up to 5 same-sex littermates in individually ventilated cages under standard specific pathogen-free conditions with a 12-hour light/dark cycle. Cages were supplied with a layer of chip bedding, one nestlet, and one puck of kraft paper strips (e.g. Bed-r'Nest or similar). Food and water were provided *ad libitum*.

2.2.1.2 Strains and Strain Maintenance

ERβ$^{fl/fl}$ mice were kindly provided by Dr. Kenneth Korach at NIH. Tg(*Cx3cr1-cre*)MW126Gsat mice (hence, *Cx3cr1-Cre)* were generated by the Heintz laboratory at Rockefeller University and were purchased from the Mutant Mouse Resource and Research Center at UC Davis. To generate *ERβ$^{fl/fl}$;Cx3cr1-Cre* mice, *ERβ$^{fl/fl}$* females were crossed to *Cx3cr1-Cre* males, generating *ERβ$^{+/fl}$; Cx3cr1-Cre* male offspring which were then backcrossed to *ERβ$^{fl/fl}$* females. To maintain breeders for experiments, *ERβ$^{fl/fl}$* mice were inbred to generate *ERβ$^{fl/fl}$* dams and *ERβ$^{fl/fl}$;Cx3cr1-Cre* males were crossed with *ERβ$^{fl/fl}$* females to generate *ERβ$^{fl/fl}$;Cx3cr1-Cre* sires. To prevent genetic drift due to inbreeding, both genetic lines were annually crossed to C57BL/6NCrl wild-type strain (Charles River Laboratories, Strain 027) for one generation, backcrossed to *ERβ$^{fl/fl}$* dams, and then maintained through inbreeding.

The stranger mice used in behavioral studies were wild type mice purchased from either Jackson Laboratories or Charles River Laboratories (C57BL/6J, Strain 000664; C57BL/6NCrl, Strain 027). Stranger mice were housed in the animal facility for at least 1 week before behavior testing. Stranger mice were sex-matched to subject mice undergoing behavioral testing, and age-matched up to 1 week older or 2 weeks younger than subject mice.

2.2.1.3 Genotyping

DNA was prepared from 1-2mm tail clips or ear punches using a quick alkaline lysis method (Truett et al., 2000). Alkaline lysis solution (25mM NaOH / 0.2 mM EDTA) was freshly made for each experiment, and 75ul was added to each tail. Tails were heated at 98°C for 20-60 min, with condensation on the lid tapped down every 10 min, and vortexed once. 75µl of 40 mM Tris HCl (pH 5.5) was added to neutralize the solution. Samples were then diluted 10-fold with water.

PCR genotyping for the *Cx3cr1-Cre* and *Esr2* floxed alleles was performed using the following general PCR reagents and concentrations (Table 2.1), as well as gene-specific primers (Table 2.2). Genotyping for *Cre* was adapted from a Qiagen Application Note that used amplification of myogenin as an internal positive control (Moullan, 2016). We redesigned and validated the primers targeting the Cre recombinase gene for more consistent amplification in our lab. Thermocycler conditions were the same for the genotyping of both *Cre* and *Esr2* floxed allele: initial denaturation at 95℃ (5 min), 32 cycles of denaturation at 95℃ (15s), annealing at 56℃ (30s), extension at 68℃ (45s), and final extention at 68℃ (5 min).

Table 2.1: PCR reagents and volumes used for genotyping Cre and Esr2 floxed allele

Reagent	**Volume**
water	11.5µl
ThermoPol Buffer (NEB #M0267S)	1.5µl
dNTPs (10mM)	0.3µl
forward primer (10uM)	0.3µl
reverse primer (10uM)	0.3µl
Taq DNA Polymerase (NEB #M0267S)	0.075µl
DNA sample	1µl
	= 15µl rxn

Table 2.2: PCR primers used for genotyping *Cre* and *Esr2* floxed allele

Target gene	Primer	Sequence	Amplicon length	Notes
Cre	F	CCCGGCAAAACAGGTAGTTA	166bp	Band = presence of Cre recombinase gene
Cre	R	CGTACTGACGGTGGGAGAAT		
Myogenin	F	TTACGTCCATCGTGGACAGC	~250bp	Control amplification added to Cre genotyping rxn
Myogenin	R	TGGGCTGGGTGTTAGCCTTA		
Esr2	F	CTGCTCCTCTGCTGGACAA	129bp	Higher band = presence of LoxP, lower band = absence of LoxP
Esr2	R	CAAGCTTCCTCTTCAGGGTCT		

2.2.1.4 Breeding for Experiments

Breeding cages were prepared after 4pm with 1-2 dams and a sire placed in a clean cage. Breeding dams were checked daily (7:00-10:00am) for vaginal plugs, and plug positive females were separated into a new clean cage, with up to one other female cage-mate. Date of plug was considered

to be E0.5. Plug positive females were weighed at E9.5-E11.5 to confirm pregnancy, and moved to a clean individual cage if not already individually housed.

ERβ$^{fl/fl}$ dams were bred with *ERβ$^{fl/fl}$;Cx3cr1-Cre* sires to generate offspring homozygous for the *ERβ* floxed allele and either positive for the Cre transgene (Cre+: experimental animals with removal of myeloid ERβ) or negative for the Cre transgene (Cre-: control littermates with myeloid ERβ intact).

2.2.2 Maternal Immune Activation

2.2.2.1 Preparation and *In Vitro* Testing of Poly(I:C)

A Poly(I:C) solution was prepared using a potassium salt containing 10% Poly(I:C) by weight purchased from Sigma-Aldrich (Cat. P9582; 50 mg total solids, >99% pure; <1% mononucleotide content). Sterile 0.8% physiological saline (0.15M NaCl) was added to make a 0.5mg/mL Poly(I:C) solution, which was aliquoted and stored at -20℃. Sterile physiological saline was also aliquoted to serve as vehicle control. After preparing the solutions, random codes were assigned to each treatment group for tube labeling so researchers doing downstream experiments were blinded to the treatment group.

The prepared Poly(I:C) solution was tested for inflammatory potency by *in vitro* stimulation of the murine microglial cell line, SIM-A9 (Nagamoto-Combs et al., 2014). RNA was extracted from the cells using TRIzol™ Reagent and the Direct-zol RNA Miniprep Kit 6 hrs after stimulation (Thermo Fisher Scientific, Cat.15596026; Zymo Research, Cat. R2050). RNA was converted to cDNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific, Cat. 18080051) and Cxcl10 mRNA levels were quantified by RT-qPCR using the KAPA SYBR® FAST qPCR Kit Master Mix (2X) ROX Low (Kapa Biosystems, Cat. KR0805) and the following primers: Cxcl10_F: CCAAGTGCTGCCGTCATTTTC, Cxcl10_R: GGCTCGCAGGGATGATTTCAA.

2.2.2.2 Injection with Poly(I:C) or Vehicle

Mice were assigned to treatment groups as follows: If multiple female mice plugged on the same day, half were randomly assigned to Poly(I:C) and half to vehicle treatment using an online list randomizer (Haahr, 1998--2022). If an odd number of females were pregnant on a day, the additional mouse was randomly assigned to Poly(I:C) or vehicle treatment group. On any following days with an odd number of females, the additional mouse was assigned to the opposite treatment group.

Aliquots of Poly(I:C) and vehicle were kept frozen, then quickly warmed in the palm 5 min before injection. Day E12.5 dams were weighed and injected at 12:00pm $\pm$ 30min. Either Poly(I:C) or vehicle was injected peritoneally using a 27 gauge needle at volume of 4ul per gram (96ul in typical 24g pregnant female). This resulted in a final dose of 2mg/kg Poly(I:C).

Table 2.3: Behavior tests and measurements conducted, in chronological order. EPM, elevated plus maze; RSI, reciprocal social interaction; Tail susp., Tail suspension test.

Behavior Tests or Measurements Conducted
Marble burying
EPM
Open field
Three chamber
Y maze
Nest height
Self-grooming/ RSI
Rotarod
Weight
Tail susp.
Forced swim

2.2.3 Behavior Testing: General Methods and Approach

A battery of 11 behavior assays was performed to assess MIA-associated phenotypes. Restricted repetitive and motor coordination behaviors were measured using the marble burying assay, spontaneous alternation in the Y-maze, rotarod, and self-grooming behavior. Sociability was measured using the three chamber sociability and social novelty assay and the reciprocal social interaction assay. Anxiety-like behavior was measured using the elevated plus maze and open field, and despair behaviors measured via the tail suspension and forced swim tests. Other general behaviors measured include activity in the open field and nest building.

Data was collected manually for some assays, or via video tracking and subsequent analysis by the Panlab SMART video tracking system (Harvard Apparatus) or by coding using the open-source software BORIS (Friard and Gamba, 2016). Data were further processed and analyzed in R to select behavior variables of interest, perform statistical testing, and plot the results.

The battery of tests performed is summarized in (Table 2.3). Details about the experimental groups are shown in (Table 2.4). The age of animals during behavior testing is summarized in (Table 2.5).

2.2.3.1 Behavior Battery General Methods

2.2.3.1.1 Timing

Each mouse had at least 3 days without testing between each behavior test (e.g. testing Monday and Friday). Mice were tested starting at 10am with habituation beginning no earlier than 9am, and testing going no later than 4pm. Time of day affects behavior, so times were kept as consistent as possible given the large cohort (Loss et al., 2015).

Table 2.4: Experimental groups and total number of animals from each group used for behavior testing.

Sex	ERβfl/fl Genotype	Cx3r1-cre Genotype	Treatment	Total Animals
F	ERβfl/fl	Cre -	PBS	5
F	ERβfl/fl	Cre -	Poly(I:C)	13
F	ERβfl/fl	Cre +	PBS	17
F	ERβfl/fl	Cre +	Poly(I:C)	16
M	ERβfl/fl	Cre -	PBS	6
M	ERβfl/fl	Cre -	Poly(I:C)	14
M	ERβfl/fl	Cre +	PBS	12
M	ERβfl/fl	Cre +	Poly(I:C)	11

Table 2.5: Animal age during behavior testing.

Age during behavior battery (months)	Age range at start of testing battery (days)	Age range at end of testing battery (days)
2-5 mo	64-77	141-154

2.2.3.1.2 Habituation

Mice were habituated to the behavior room or suite for 20-30 min prior to testing, depending on the test and specified in individual methods sections below. Mice were habituated to the testing room in their home cages and remained in the testing room during testing of other mice for the open field, marble burying, EPM, Y-maze, and self-grooming. Mice were habituated to the behavior suite hallway in their home cages and remained in the hallway during the testing of other mice for the RSI and 3 chamber test. Mice remained in a separate room for the forced swim and tail suspension tests to prevent excess stress from hearing other mice undergoing the test.

2.2.3.1.3 Caging

Mice were singly housed in individual fresh cages containing bedding and nest materials for 10 min prior to test start (with the exception of open field, three chamber, and marble burying which include a habituation period in the test). Mice were temporarily singly housed again after testing, and returned to home cages when all cage-mates completed testing.

2.2.3.1.4 Testing environment

Each testing apparatus was cleaned with 70% EtOH before each trial. At least 5 min between cleaning and the start of the next trial was allowed for ethanol evaporation and odor dissipation. Males and females were tested on separate days, and behavior room air was allowed to vent by

leaving the door open between testing of different sexes. The researcher was absent from the testing room during trials, with the exception of the rotarod test.

2.2.3.1.5 Video Recording and Data Analysis

Most behavior tests were monitored and recorded using a Logitech C270 webcam. SMART Video Tracking System (Panlab) software from Harvard Apparatus was used for analysis in real time for all behaviors except three chamber, reciprocal social interaction, self-grooming, marble burying, and nest height. Three chamber videos were recorded and later analyzed by SMART software. Reciprocal social interaction and self-grooming videos were manually scored using the open-source BORIS behavior coding software. Data were then imported into RStudio (v2022.12.0) for further processing, statistical analysis, and visualization using the R language (v4.2.1) (R Core Team, 2022; RStudio Team, 2022). The core tidyverse packages (v1.3.2) and the here package (v1.0.1) were used to import, clean, and process data, (Wickham et al., 2019; Müller, 2020), tables were formatted using the knitr (v1.40), kableExtra (v1.3.4), and gt (v0.8.0) packages (Zhu, 2021; Iannone et al., 2022; Xie, 2022), the ggbeeswarm (v0.6.0) and scales (v1.2.1) packages were used in addition to ggplot2 for plotting (Clarke and Sherrill-Mix, 2017; Wickham and Seidel, 2022), and statistical analyses were performed using the R stats (v4.2.1), limma (v3.54.0), multcomp (1.4.20), broom (v1.0.1), and rstatix (0.7.1) packages (Hothorn et al., 2008; Ritchie et al., 2015; Kassambara, 2022; Robinson et al., 2022). Data from each assay was fit to a linear model followed by multiple planned comparisons and adjustment of p-values using Benjamini Hochberg correction (further explanation of statistical tests is in the Results section).

2.2.4 Behavior Testing: Individual Test Methods

2.2.4.1 Elevated Plus Maze

Mice were habituated to the testing room in their home cages for at least 20min prior to testing. The elevated plus maze had two open arms and two closed arms, each 30 × 5 cm, and a central platform area, 5 × 5 cm. Mice were placed in the center of the elevated maze and allowed to freely explore for 10 min. Time spent in the open arms, closed arms, and center zone, and number of entries into open arms and closed arms were measured.

2.2.4.2 Forced Swim

Mice were habituated in a separate suite from the testing room in their home cages for x 30 min. Two clear plastic cylinders (20cm diameter, 45cm tall, with base) were filled halfway with 25 ± 0.5 ℃ tap water measured with a thermometer. The cylinders were placed on a cart, with an opaque visual barrier between them. Two mice were tested at a time: both mice were brought into the test room in their individual cages, each placed into the water in a cylinder, then recorded for a 6 min trial. After the trial, mice were returned to individual cages for recovery for at least 20 min. Recovery cages were placed half way onto a heating pad in the habituation room (to allow

mice to choose a warm or ambient side of the cage). Cylinders were rinsed and filled with fresh water each trial. Activity was scored using the SMART video software with threshold settings of 40cm^2/s for low activity and 200cm^2/s for high activity, and detection settings at threshold of "10." Animals were considered immobilized when activity was below the low activity threshold.

2.2.4.3 Marble Burying

Mice were habituated to the behavior room in their home cages for x 30min prior to testing. Each mouse was placed individually in a cage ~40% filled with bedding and allowed to habituate for 20min with the lid on. Mice were then removed and placed in an individual fresh cage while bedding in the test cage was leveled, patted flat, and marbles placed on top in 5 x 4 grid (Figure 2.1). Mice were then placed back in their original test cage with lid on and allowed to freely move for 20 min. At the end of the trial, mice were moved back to their individual fresh cages until all mice completed the test, then they returned to their home cage. Two measurements were recorded at the end of the trial: 1) The total number of incompletely buried marbles (meaning any that are at all visible), and 2) the number of marbles <½ buried, meaning more than half the marble was visible. These numbers were then subtracted from 20 to get two metrics for analysis: 1) the number of fully buried marbles, and 2) the number of marbles >½ buried. A photo of the cage surface was taken for validation of measurements. Up to five cages were tested at once, spaced >10cm apart on a table top or cart.

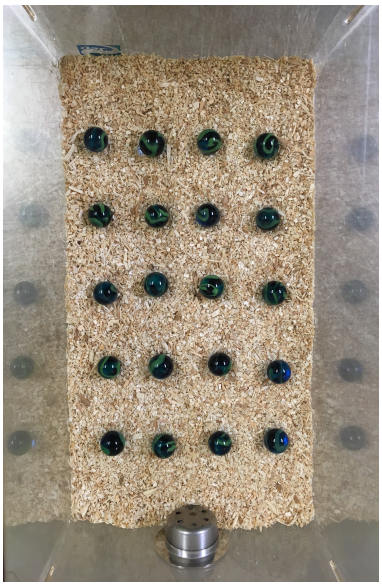


Figure 2.1: Arrangement of marbles in the cage bottom for the marble burying test.

2.2.4.4 Nest Height Measurement

Mice were placed in fresh individual cages with a layer of bedding, one nestlet, and one 8g puck of kraft paper strands (typical nesting material that mice had previously encountered in their home cages). Mice remained in the cage for x 6 days before nest height was measured. Nests were carefully removed from the cage and placed on a clean surface, and the 5th highest point of the nest was measured (to counteract outliers from a single high strand). Nests were then returned to the cage.

2.2.4.5 Open Field

The open field chambers consisted of white sides and blue floor, measuring approximately 40 cm wide × 40 cm long × 30.5 cm deep. Four chambers were positioned adjacent to one another in a larger square with an overhead camera, allowing for a maximum of four mice to be tested at once. Mice were habituated to the behavior room in their home cage for x 30 min prior to testing. Mice were placed in the center of the open field at the initiation of the testing session. After all mice were placed, the 30 min trial was started. Several measurements were collected, including total distance traversed and time spent in the periphery or the center of the field. After the trial, mice were removed into fresh cages until all cage-mates were tested, then returned to their home cage. The number of defecations (pellets in the open field arena at the end of the test) were manually counted.

2.2.4.6 Reciprocal Social Interaction

The reciprocal social interaction (RSI) test was adapted so that self-grooming and other behaviors could be measured during the RSI habituation time.

Mice were separated into individual cages 7 days before testing. Prior to testing, mice were habituated to the behavior suite hallway in their home cages for x 20 min. Immediately before testing, the cage insert containing food and water, the nest, and the lid were removed from the test cage. The cage was placed on a platform to enable video recording from both the top and side angles. The first 10 min of the trial were recorded to measure self-grooming behavior. Next, a wild-type age- and sex-matched stranger mouse was placed in the corner of the test cage and interactions were recorded via overhead camera for 5 min. The stranger mouse was visually differentiated from the subject mouse by clipping hair off a section of the stranger mouse's back, creating a bald patch visible on video. Stranger mice were returned to their home cage after trials, and allowed to rest for at least 2 trials before acting as a stranger mouse again that day. Stranger mice rested for 2 days before serving as stranger mice on another day.

2.2.4.6.1 Video Scoring and Analysis

Videos were scored manually using Solomon Coder or BORIS open-source software. This enabled more detailed coding of specific behaviors, listed in (Table 2.6). Analysis of grooming behaviors was adapted from the categories identified by Kalueff et al. (2007).

Videos were first transcoded to a readable format (.mp4) using the open-source software Handbrake, then opened in the behavior coding software. In cases where two videos were recorded simultaneously, split-screen videos were created or both videos played simultaneously in BORIS (longer video in player 1 and second video adjusted so times match). Data was exported as a csv and loaded into R for further analysis and visualization. The ICC R package was used to test for inter-rater reliability (Wolak et al., 2012).

Table 2.6: Behaviors coded from video of the reciprocal social interaction test. Behaviors are categorized as grooming behaviors, social interaction behaviors, or other. The type of behavior is defined as either a "Point" or "State" event, indicating a behavior without duration, or an ongoing behavior with duration for which start and stop times were coded, respectively.

Behavior code	Behavior type	Behavior category
Paw/nose/face	State event	Grooming
Head/ear	State event	Grooming
Body	State event	Grooming
Tail/genitals	State event	Grooming
Aggression	State event	Interaction
Mounting	State event	Interaction
Ano-genital sniffing	State event	Interaction
Body sniffing	State event	Interaction
Nose-nose sniffing	State event	Interaction
Push-crawl	State event	Interaction
Front approach	State event	Interaction
Rearing on wall	Point event	Other
Rearing stand	Point event	Other
Digging	State event	Other
Climbing	State event	Other

2.2.4.7 Rotarod

A 5-lane rotating rod was used to measure motor coordination, allowing up to five mice to be tested simultaneously (RotaRod, Ugo Basile Cat. 47650). Before testing each day, mice were habituated to the testing room for x 30 min. On the first day of testing, mice were acclimated to the apparatus by walking on the rod while it rotated at 5 RPM. Mice continued to walk until they stayed on the rod continuously for 60 s, indicating they had adjusted to the apparatus when

walking at this slow speed. For the trials, the apparatus was programmed for a ramp setting with an initial speed of 5 RPM, final speed of 80 RPM, and 300 s ramp, and mice were observed for a maximum of 5 min. Latency to spin (i.e. stop walking and instead grip on the rod and spin around) and latency to fall were both recorded. Mice that were spinning for ~15 s or more were gently lifted and set back on the rod. After a trial, mice were placed in individual holding cages for a 5-7 min inter-trial period before the next trial. This was repeated for 3 trials per day over 4 consecutive days for a total of 12 trials. RPM at first spin or fall was then calculated as follows: latency*75/300+5.

2.2.4.8 Tail Suspension

The tail suspension test (TST) was performed following a previously described protocol (Can et al., 2011). A 4-chamber apparatus with a rod running along the top was used for the tail suspension test (chambers were open on one side to allow recording of the test but dividers prevented mice from seeing mice in neighboring chambers). Mice were habituated in a separate room from the testing room in their home cages for x 30 min. Strips of 17cm tape were prepared, with a line marked 2 cm from the end. A plastic cylinder was placed around the base of each mouse's tail to prevent climbing up the tail, and the 2cm end of the tape was wrapped around the end of the tail. The mice were taped at the bottom of the apparatus until all mice were ready, then one by one suspended from the bar using the tape. A 6 min trial was recorded and activity was scored in real-time using the SMART video software. After the trial, mice were returned to home cages, or housed in a holding cage with cage-mates until all mice from their home cage finished with behavior testing and then returned to their home cages. Activity was scored using the SMART video software with threshold settings of 30cm^2/s for low activity and 110cm^2/s for high activity, and detection settings at threshold of "15."

2.2.4.9 Three Chamber Sociability and Social Novelty

A plexiglass apparatus containing 3 rectangular chambers connected by small doorways was used (Figure 2.2). Mice were habituated to the behavior suite hallway for x 20 min prior to testing. Mice were placed in the center chamber of the three chamber apparatus and allowed to freely explore for 10 min. At ~9 min into the test, outside the testing room, a stranger mouse was inserted into the S1 (stranger 1) cylinder. The test mouse was then shepherded into the center chamber and plastic doors were inserted to contain the mouse in the center chamber. The S1 cylinder was placed in the left chamber and the E (empty) cylinder was placed in the right chamber. The doors were opened and the mouse was allowed to freely explore for 10 min. Cylinders were removed, followed by once more shepherding the mouse into the center and inserting the doors. A second stranger mouse was then inserted into the E cylinder, now labeled S2 (stranger 2), and cylinders were placed again in the same locations, followed by door opening. The mouse was allowed to freely explore for 10 min. The entire 30min test was recorded using an overhead camera, and later analyzed using SMART tracking software. After the test, subject mice were placed in fresh holding cages until all litter-mates being tested that day were finished, then returned to

their home cage. Measurements include time spent in each chamber as well as time spent in the circular area surrounding each cylinder (within ~4 in of the cylinder).

Figure 2.2: Set up for the Three Chamber Social Interaction test showing apparatus with cylinders.

2.2.4.10 Weight

Mice were individually weighed by placing them in a plastic cup on a scale at the conclusion of rotarod trials and measuring mass in grams. Mice were 4-5 months old at time of measurement.

2.2.4.11 Y-Maze Spontaneous Alternation

The Y-maze apparatus was made of dark grey, opague plexiglass and consists of 3 enclosed arms of equal length oriented at 120° angles around a small central triangular chamber. Mice were habituated to the testing room in their home cages for x 20 min, and then habituated to an individual cage for 10 min prior to testing in the Y-maze. Doors were inserted at the entry to each arm of the maze and the test mouse was inserted into the center space between doors. Doors were then removed and the mouse was allowed to freely explore for 10 min. Trials were recorded by overhead camera and later analyzed using the Panlab SMART tracking software. “Alternation” was defined as entering each of three arms sequentially without repeating an arm, and “spontaneous alternation” was calculated as the total number of alternations divided by the total arm entries minus 2.

Table 2.7: The number of animals from each experimental group used in behavior assays.

Sex	Treatment	Cre Genotype	n
Female	Veh	Cx3cr1-cre -	5
Female	Veh	Cx3cr1-cre +	17
Female	Poly(I:C)	Cx3cr1-cre -	13
Female	Poly(I:C)	Cx3cr1-cre +	16
Male	Veh	Cx3cr1-cre -	6
Male	Veh	Cx3cr1-cre +	12
Male	Poly(I:C)	Cx3cr1-cre -	14
Male	Poly(I:C)	Cx3cr1-cre +	11

2.3 Results

2.3.1 Female and Male MIA Offspring Were Tested in a Behavioral Battery Relevant to Maternal Immune Activation Phenotypes

We sought to investigate the impact of ERβ in myeloid cells on the response to maternal immune activation (MIA). We assessed the behavioral phenotypes of *ERβ$^{fl/fl}$;Cx3cr1-Cre* female and male offspring and their *ERβ$^{fl/fl}$* control littermates after exposure to Poly(I:C) MIA or vehicle. For this behavioral study, dams were injected intraperitoneally with 2mg/kg Poly(I:C) or vehicle at E12.5, and left undisturbed through birth and until weaning of offspring at 21-28 days. Offspring were housed with age- and sex-matched cagemates. We evaluated the behavior of 40 offspring from 6 vehicle-treated litters and 54 offspring from 9 Poly(I:C)-treated litters.

2.3.2 Specific Statistical Comparisons Between Experimental Groups were Designed to Limit Multiple Hypothesis Testing

Variability in phenotypes is well-documented in MIA models as well as animal behavior testing (Becker et al., 2016; Robinson et al., 2018; Estes et al., 2019; Rubio Arzola and Shansky, 2022). Given this variability and differences in sample size, we fit data from each assay to a linear model using `lm` from the stats package followed by multiple planned comparisons using `glht` from the multcomp package (Hothorn et al., 2008; R Core Team, 2022). We implemented a few methods to account for multiple hypothesis testing, given the multi-factorial design of the study (Sex, Genotype, and Treatment factors) and collection of data from multiple behavior assays. First, we specified specific contrasts of interest to test rather than testing all pairwise comparisons between groups (Table 2.8). We followed up with additional treatment-related comparisons only if there was a significant overall effect of Poly(I:C) treatment or of Poly(I:C) treatment in *ERβ$^{fl/fl}$* (cre negative) males or females at $p < 0.05$. Following these methods, we made 132 initial comparisons (6 comparisons each for 15 behavior variables of interest and 7 control variables) and 24 follow-up

comparisons. We compiled results from these tests and used the Benjamini Hochberg correction to adjust p-values for multiple comparisons, resulting in 7 significant comparisons at $p < 0.10$ (Table 2.9). One of these was the expected sex difference in weight.

2.3.3 Males Display Increased Marble Burying Behavior

We found an overall sex difference in marble burying behavior. There was a significant difference in the number of marbles over halfway buried (adjusted $p = 0.000124$), with females (mean = 6.7, sd = 5.3) burying fewer marbles than males (mean = 13.4, sd = 5.0) (Figure 2.3). A similar sex difference was found using a different metric, when counting number of marbles fully buried (adjusted $p = 0.0965$) (Figure 2.4).

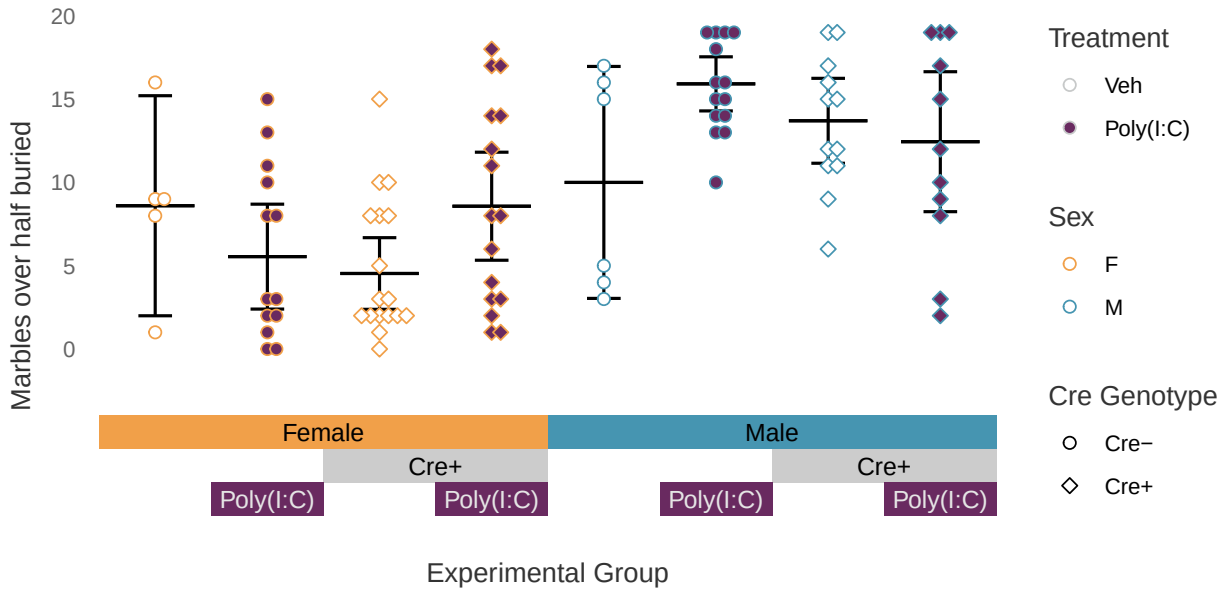


Figure 2.3: Number of marbles over half buried during the 20 min marble buring test. Trimmed mean for each group is shown with a horizontal bar and 95% confidence intervals are shown with error bars. The legend to the right decribes the symbols used for experimental groups.

Table 2.8: Contrasts used to compare coefficients in the linear model for each behavior variable. Coefficients estimate the average behavior variable value for each group. The research question motivating a given contrast is listed, as well as the testing group. Testing group 1 comparisons were tested for all variables, while testing group 2 comparisons were made conditional on the results from the first round of testing. Also included are the abbreviation for the contrast used in reporting significant differences for that contrast and the mathematical formula comparing the coefficients to generate each contrast.

Research Question	Testing Group	Contrast	Comparisons Between Coefficients
Effect of Poly(I:C) in Cre- Males	1	M_CreNeg_VehvsPolyIC	Male Veh Cre- - Male Poly(I:C) Cre-
Effect of Poly(I:C) in Cre- Females	1	F_CreNeg_VehvsPolyIC	Female Veh Cre- - Female Poly(I:C) Cre-
Overall effect of Poly(I:C)	1	VehvsPolyIC	Sum of all vehicle groups/4 - Sum of all Poly(I:C) groups/4
Overall effect of Sex	1	FvsM	Sum of all female groups/4 - Sum of all male groups/4
Effect of Cre genotype in Veh Males	1	M_Veh_CreNegvsCrePos	Male Veh Cre- - Male Veh Cre+
Effect of Cre genotype in Veh Females	1	F_Veh_CreNegvsCrePos	Female Veh Cre- - Female Veh Cre+
Differential effect of Poly(I:C) by Sex in Cre-	2	CreNeg_Tx_FvsM	(Female Veh Cre- - Female Poly(I:C) Cre-) - (Male Veh Cre- - Male Poly(I:C) Cre-)
Differential effect of Poly(I:C) by Cre Genotype in Males	2	M_Tx_CreNegvsCrePos	(Male Veh Cre- - Male Poly(I:C) Cre-) - (Male Veh Cre+ - Male Poly(I:C) Cre+)
Differential effect of Poly(I:C) by Cre Genotype in Females	2	F_Tx_CreNegvsCrePos	(Female Veh Cre- - Female Poly(I:C) Cre-) - (Female Veh Cre+ - Female Poly(I:C) Cre+)

Table 2.9: Significant comparisons from behavior battery. The particular behavior variable and group contrast used for each comparison are listed, with associated p value and adjusted p value after Benjamini Hochberg correction. Comparisons with adjusted p values < 0.10 are included.

Behavior variable	p value	p value (adj)	Contrast
Weight in grams	0.0000000	0.0000000	FvsM
Marbles over half buried	0.0000016	0.0001243	FvsM
Total Distance (EPM)	0.0003029	0.0157505	F_CreNeg_VehvsPolyIC
Total Distance (EPM)	0.0007601	0.0296458	VehvsPolyIC
Time Immobile (Tail Susp.)	0.0016783	0.0523645	F_Tx_CreNegvsCrePos
Time Immobile (Tail Susp.)	0.0029269	0.0760996	F_Veh_CreNegvsCrePos
Marbles fully buried	0.0043304	0.0965052	FvsM

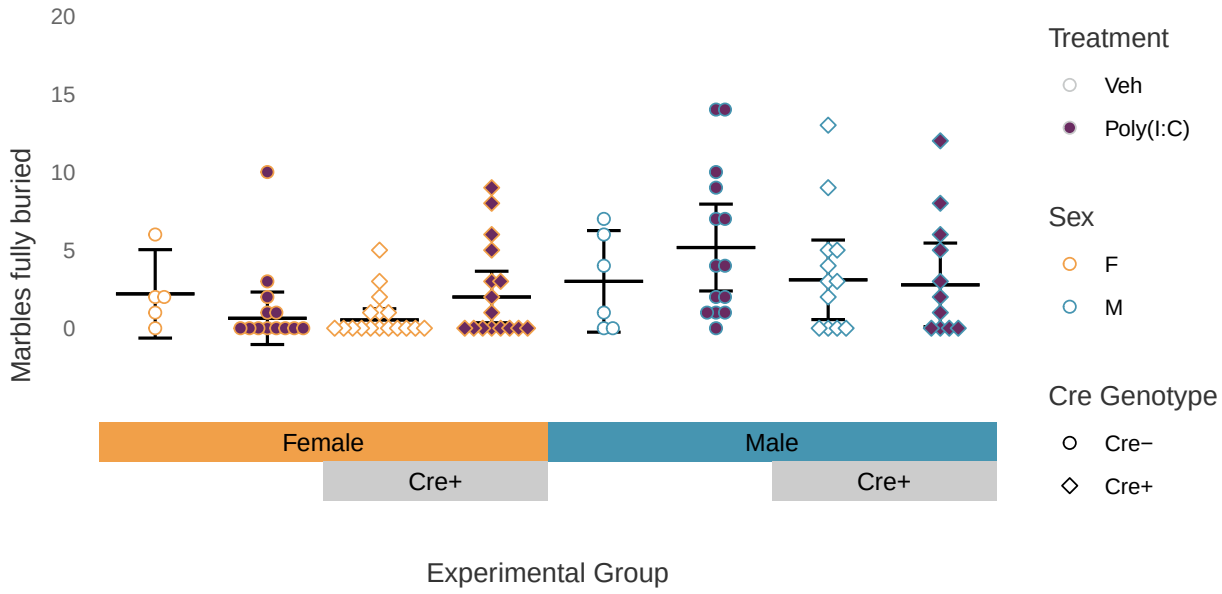


Figure 2.4: Number of marbles fully buried during the 20 min marble buring test. Trimmed mean for each group is shown with a horizontal bar and 95% confidence intervals are shown with error bars. The legend to the right decribes the symbols used for experimental groups.

2.3.4 *ERβ$^{fl/fl}$;Cx3cr1-cre* Genotype and Poly(I:C) Treatment May Impact Immobility Time in the Tail Suspension Test

The tail suspension test measures despair behavior and time spent immobile is thought to reflect depression-like phenotypes. We found a difference in immobility time between *ERβ$^{fl/fl}$;Cx3cr1-cre*

and $ER\beta^{fl/fl}$ genotype groups among female vehicle-treated mice (adjusted p = 0.0760, Figure 2.5). $ER\beta^{fl/fl}$;*Cx3cr1-cre* mice spent less time immobile (mean = 33.5s, sd = 13.6) compared to control $ER\beta^{fl/fl}$ litter mates (mean = 54.1s, sd = 10.5). There was also a difference in Poly(I:C) effect based on genotype in female mice in the tail suspension test (adjusted p = 0.0524). For females with $ER\beta^{fl/fl}$;*Cx3cr1-cre* genotype, Poly(I:C)-treated mice (mean 45.7, sd = 11.6) had increased average immobility time relative to vehicle-treated mice (mean = 33.5, sd = 13.6). However, for females with $ER\beta^{fl/fl}$ genotype, Poly(I:C)-treated mice (mean = 39.3, sd = 11.6) had decreased average seconds of immobility relative to vehicle-treated mice (mean = 54.1, sd = 10.5).

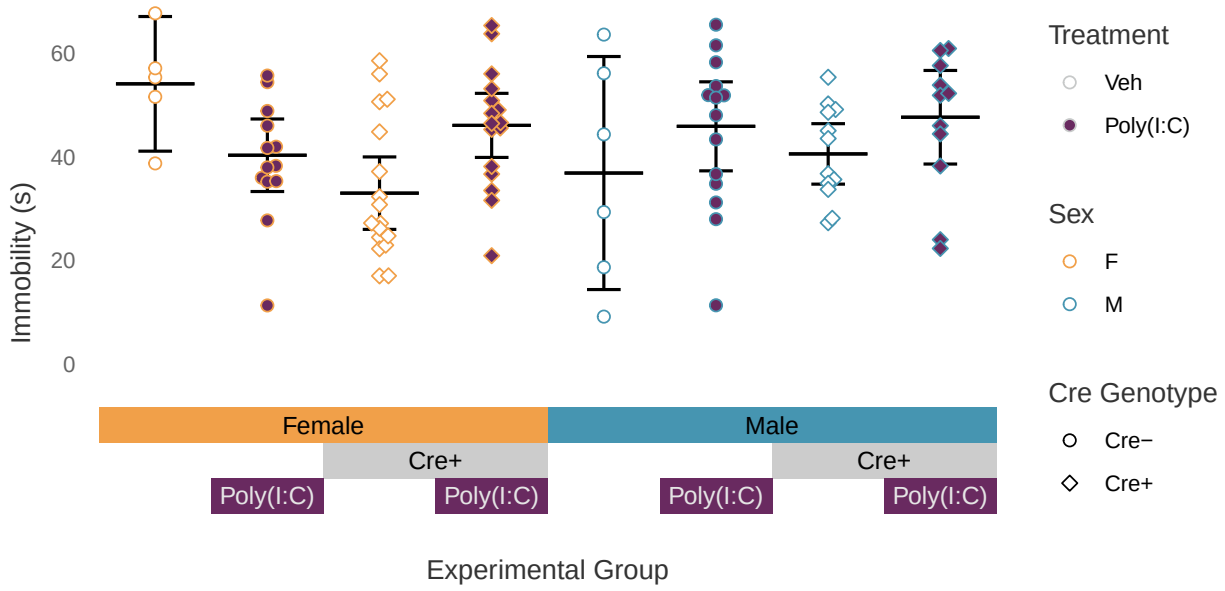


Figure 2.5: Seconds spent immobile during the tail suspension test. Trimmed mean for each group is shown with a horizontal bar and 95% confidence intervals are shown with error bars. The legend to the right decribes the symbols used for experimental groups.

2.3.5 Poly(I:C)-Treated Mice Travel Shorter Distances in the Elevated Plus Maze

Surprisingly, we also saw differences between groups in one of the control variables, total distance traveled in the elevated plus maze (Figure 2.6). There was an effect of treatment within the $ER\beta^{fl/fl}$ female mice, in which the Poly(I:C) treated group had lower average total distance compared to the vehicle group (adjusted p = 0.0157). There was also an overall treatment effect when comparing average distance across all Poly(I:C) mice with all vehicle treated mice, in which mice exposed to Poly(I:C) traveled shorter distance (adjusted p = 0.0296).

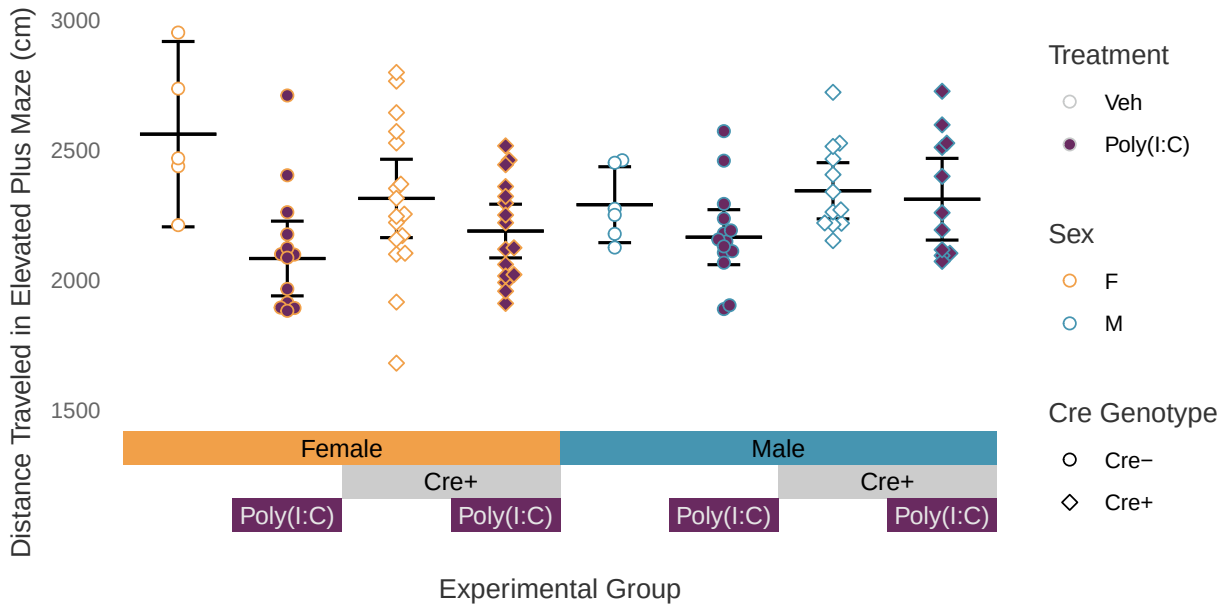


Figure 2.6: Total distance traveled in cm during the elevated plus maze assay. Trimmed mean for each group is shown with a horizontal bar and 95% confidence intervals are shown with error bars. The legend to the right decribes the symbols used for experimental groups.

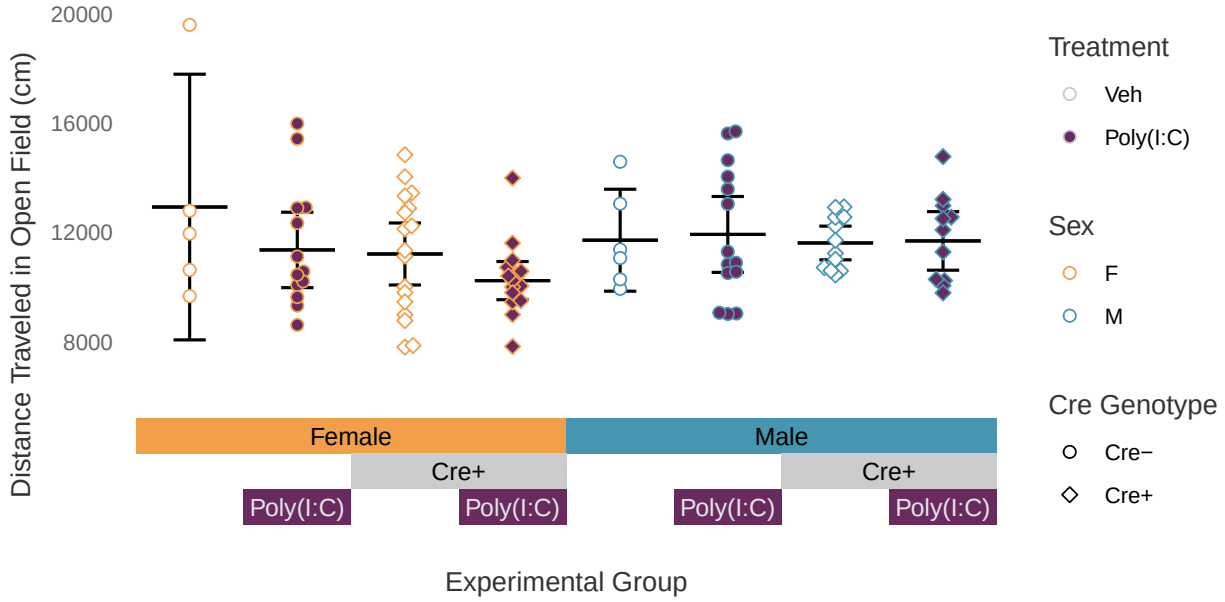


Figure 2.7: Total distance traveled in cm during the elevated plus maze assay. Trimmed mean for each group is shown with a horizontal bar and 95% confidence intervals are shown with error bars. The legend to the right decribes the symbols used for experimental groups.

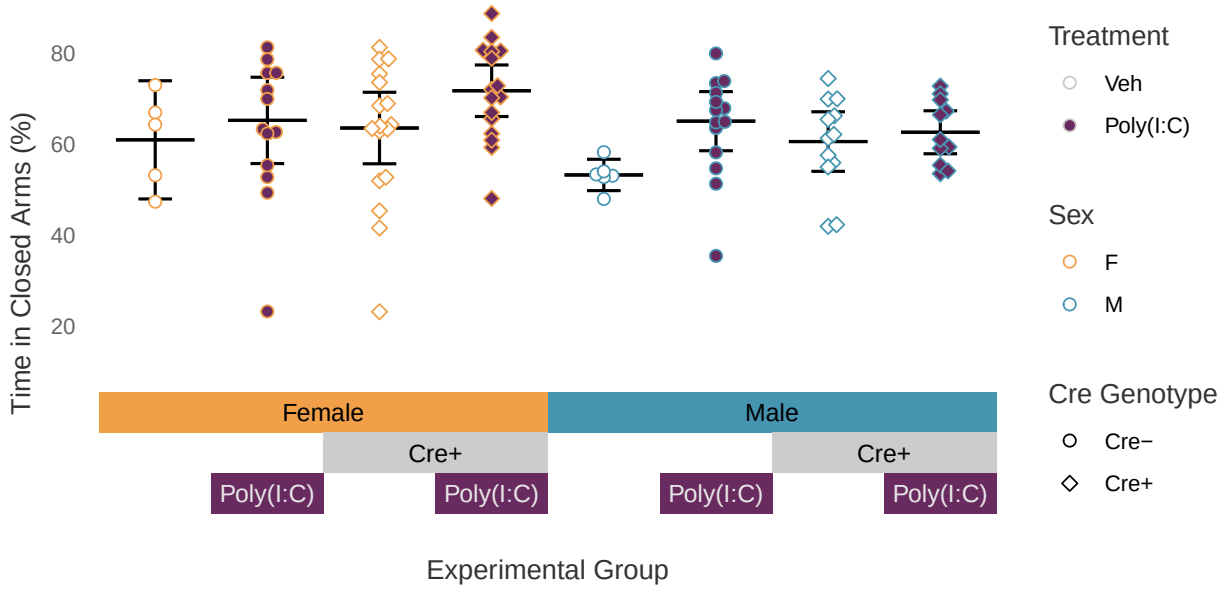


Figure 2.8: Percent of time spent in the closed arms during the elevated plus maze assay. Trimmed mean for each group is shown with a horizontal bar and 95% confidence intervals are shown with error bars. The legend to the right decribes the symbols used for experimental groups.

2.4 Discussion

2.4.1 Sources of Error and Variability in Behavior Testing

We observed few significant differences in behavior between experimental groups in this study. Behavioral assays used frequently in the literature to measure animal phenotypes relevant to the core symptoms of autism include marble burying (restricted, repetitive behavior), and the three chamber test and reciprocal social interaction test (social interaction). MIA induced by Poly(I:C) typically leads to reduced social interaction and increased marble burying in male offspring (Malkova et al., 2012; Choi et al., 2016) . We did not find the expected effect of Poly(I:C) treatment on these behaviors, making it difficult to draw conclusions about our hypothesized regulation of the Poly(I:C) effect by ERβ in myeloid cells.

There are a few possibilities that might explain why we did not observe the expected decrease in sociability and increase in marble-burying in the Poly(I:C) treated offspring compared to vehicle controls. First, it's possible that the Poly(I:C) induction of maternal/fetal immune activation was unsuccessful. We used a lower dose (2mg/kg) than typically used in the literature (20mg/kg) based on advice from members of our lab who observed high levels of pregnancy loss with the 20mg/kg dose in our lab setting. In the next chapter, in which we investigate the embryonic brain transcriptome in response to MIA, we assess whether the maternal immune response is activated

by different Poly(I:C) doses by examining maternal pregnancy outcomes and sickness phenotypes. Future studies using MIA models should be sure to validate the model in each laboratory setting and for each researcher to determine an appropriate dose for the phenotypes of interest (Kentner et al., 2018).

Another potential reason for the absence of significant Poly(I:C)-induced behaviors could be low sample size and high variability, reducing the power of statistical tests. Unequal sample sizes result from different sources of drop out in the study; for example, plug positive dams are not pregnant, pregnancy is spontaneously aborted, offspring are of an uneven sex distribution, or small litter size. The low sample sizes for $ER\beta^{fl/fl}$ vehicle-treated females (n = 5) and males (n=6) due to these random drop out effects reduced the power of our study. The researcher was blinded to both treatment and genotype, so among the 94 offspring tested it was unexpected to have such low sample size for two groups, which was not clear until the analysis stage. In future, we recommend doing a power analysis as well as determining the likelihood of given sample sizes based on the breeding and treatment experimental design in order to decrease the chance of low sample sizes in particular groups. Alternatively, blinding procedures could include steps to ensure equal sample sizes before behavior testing.

Variability in measures of animal behavior is common, since behaviors are often influenced by genetic strain differences, inter-individual variability, and environmental factors such as lighting, researcher gender, housing condition and more (Rasmussen et al., 2011; Armario and Nadal, 2013; Sorge et al., 2014; Butler-Struben et al., 2022). Social behavior specifically has been shown to be influenced by estrous cycle in females, and social dominance hierarchy in males (Kunkel and Wang, 2018; Chari et al., 2020). These factors may have contributed to variability in the behavioral results we collected, reducing the ability to detect differences between experimental conditions. In MIA models a number of additional sources of variability have been reported, including baseline level of maternal immune responsiveness and variability by Poly(I:C) reagent batch (Kowash et al., 2019; Estes et al., 2020). Jiménez and Zylka (2021) suggest using litter as a biological unit of replication or otherwise controlling litter effects to improve rigor when assessing impact of treatments during gestation. Both between- and within- litter effects were shown in a large study of Poly(I:C) MIA offspring (Mueller et al., 2020). Interestingly, when grouping offspring into "resilient" and "susceptible" groups, around half the Poly(I:C)-treated offspring displaying MIA-associated behaviors and around half displayed behavior similar to controls. This lack of phenotypic penetrance in the model also reduces power to detect Poly(I:C) effects as well as uncover the impact of additional factors on MIA phenotypes. Intriguingly, in a rat prenatal valproic acid model of neurodevelopmental disorders (NDD), environmental enrichment reversed many of the valproic acid-induced behaviors, suggesting simple environmental variables have important consequences for behaviors relevant to NDD research (Schneider et al., 2005). Together, these studies point to important sources of variation to attempt to control or model in future studies. For example, researchers have made suggestions for improving consistency in the marble burying assay and MIA models (Brouwer et al., 2018; Estes et al., 2019).

2.4.2 Sex Differences in Marble Burying

Despite individual behavior variability and concerns about statistical power, we did observe some significant differences between experimental groups. First, we saw a sex difference in marble burying, with females overall burying fewer marbles than males. As previously mentioned, behavioral studies accounting for sex differences are historically and currently under-researched, including those using the marble burying assay. Studies examining marble burying in male and female mice did not report baseline sex differences (Taylor et al., 2017; Mori Ayub et al., 2022). However, sex differences in marble burying in response to S-ketamine were observed, with females requiring a larger dose to see reduction in marble burying behaviors (Mori Ayub et al., 2022). Sex differences were also observed when stratifying mice according to their level of fear extinction (Emtyazi et al., 2022). These studies suggest that sex differences in marble burying behavior can appear when considering a modifying variable.

The source of the sex difference we observed in marble burying in our study is unknown, though further investigating baseline sex differences in this assay will be important since it is often used as a measure of autism-like and obsessive-compulsive-like behavior. Differences in female behavior are sometimes suggested to be caused by estrous cycle effects, though the impact of estrous stage on female marble burying behavior is unclear. Emtyazi et al. (2022) examined marble burying in female mice categorized by estrous stage and found no effect of estrous cycle on marble burying, while Llaneza and Frye (2009) saw an effect of estrous cycle and progesterone and/or estrogen administration in female rats, though total marbles buried in this study was small. It could be informative to stratify females according to estrus cycle to examine the effect on marble burying and variability. Whether or not the specific mechanisms causing sex differences in marble burying in a particular laboratory environment is experimentally investigated, the fact that behavior outcomes may differ by sex suggests researchers should account for potential sex differences when running the marble burying assay, potentially using different metrics, set-up, or length of time for the test in order to capture the range of phenotypes in both females and males.

2.4.3 Impact of Genotype and Poly(I:C) Treatment on Immobility in the Tail Suspension Test in Females

We observed female-specific impacts of genotype and Poly(I:C) treatment in the tail suspension test (TST), a measure of depression-like behavior. *ERβ$^{fl/fl}$;Cx3cr1-cre* female mice had reduced depression-like behavior compared to *ERβ$^{fl/fl}$* female mice as measured by immobility in the TST. On the other hand, we observed no Cre genotype-mediated effect in males. These data suggest that *ERβ* in myeloid cells may be important in expressing baseline levels of despair behavior in females, and Cre-mediated ablation of *ERβ* in myeloid cells may reduce naturally occurring depression-like phenotypes in females. This reduced immobility time in *ERβ$^{fl/fl}$;Cx3cr1-cre* animals was unlikely due to general hyperactivity, since distance traveled in the open field and elevated plus maze was not higher in *ERβ$^{fl/fl}$;Cx3cr1-cre* females relative to *ERβ$^{fl/fl}$* females.

We also observed a *Cx3cr1-cre* genotype-dependent effect of Poly(I:C) on immobility time in females. In control mice, Poly(I:C) treatment was associated with reduced immobility, whereas in *Cx3cr1-cre* mice, Poly(I:C) was associated with increased immobility. Together, these data suggest that loss of *ERβ* in myeloid cells could lead to reduced immobility time in the absence of prenatal immune insult, but higher susceptibility to increases in immobility time in the presence of MIA. Effects of Poly(I:C) and LPS MIA on depression-like behavior has been reported previously, with MIA offspring demonstrating increased immobility in the tail suspension and forced swim tests, however only male mice were examined in these studies (Khan et al., 2014; Depino, 2015). Our results indicate that the directionality of response to MIA in females in regards to immobility in the TST may be impacted by sex hormone pathways in myeloid cells. These results highlight the importance of including females in studies of depression-like behavior after MIA, since these behaviors may arise through distinct mechanisms.

In humans, females are overall more susceptible to depression (Debra J. Brody, M.P.H., Laura A. Pratt, Ph.D., and Jeffery P. Hughes, M.P.H, 2019). Reports of sex differences in depression-like behavior in pre-clinical models have been inconsistent, sometimes reporting more or less severe phenotypes in females, or no difference between females and males (Pitzer et al., 2022). Pitzer et al. (2022) sought to investigate sources of laboratory-based variability by examining female and male mice from multiple mouse strains in assays for despair-like, apathy-like, and anhedonia behaviors. Like our study, they found no baseline sex differences in depression-like behavior in the tail suspension test (TST). However, they found differences in the TST depending on mouse strain, and also strain-dependent sex differences in grooming behavior in a splash test. Similar to the effects observed in our study, this study suggests the importance of genetic factors in modulating depression-like behaviors as well as in modulating sex differences in those behaviors in rodents. Environmental stimuli, such as chronic mild stress, have been linked to depression-like behavior in animal models, and this stress paradigm may result in sex differences in behavior (Jiang et al., 2022).

The combined effects of a genetic alteration (*ERβ$^{fl/fl}$;Cx3cr1-cre* genotype), an environmental insult during development (Poly(I:C) treatment), and sex effects may be interacting in complex ways to produce the observed group differences in depression-like behavior. We hypothesized that *ERβ$^{fl/fl}$;Cx3cr1-cre* females would be protected from Poly(I:C)-induced depression-like behavior, and we did see a difference in the direction of effect of Poly(I:C) depending on genotype in females. However, the presence of baseline differences between *ERβ$^{fl/fl}$;Cx3cr1-cre* and *ERβ$^{fl/fl}$* in the vehicle-treated condition make it difficult to interpret the results in a straightforward manner.

Given the time between maternal immune activation and behavior testing, the developmental time period when the absence of *ERβ* in myeloid cells exerts effects to influence despair behavior is unclear. In humans, depressive symptoms often appear in young adulthood, and hormonal changes that occur with puberty could be involved in onset (Angold et al., 1999; Solmi et al., 2021). Myeloid ERβ could be responding to sex hormones in puberty to modulate depression-related phenotypes. One way to investigate this question is to study the behavior of juvenile mice in addition to adult mice. A inducible knock down of ERβ in myeloid cells could also shed light on the question of which developmental processes are important for the observed differences in depression-like behavior.

2.4.4 Effect of Poly(I:C) on Behavior in the Elevated Plus Maze

Finally, we observed an unexpected decrease in distance traveled in the elevated plus maze (EPM) in Poly(I:C)-treated animals compared to vehicle-treated across sex and genotype groups. This reduced locomotion appeared to be EPM-specific, since we didn't see the same overall reduction in the open field assay. When examining other data from the EPM, there was a trend toward more time spent in the closed arms in the Poly(I:C)-treated groups (un-adjusted $p = 0.0237$). From observing video recordings, mice often spent time standing still or grooming in a single spot in the closed arms. Such behavior would result in lower total distance traveled than mice actively exploring the maze. Whether the lower distance traveled in the EPM after Poly(I:C) represents an anxiety-like behavior is hard to ascertain, since total distance is often used as a control variable. Additional assays measuring anxiety, such as the light-dark box or latency to approach novel objects could help determine if Poly(I:C) has an effect on anxiety-like behaviors. Given the well-documented hyper- and hypo-sensitivities to sensory stimuli present in ASD, it's also possible that behavior changes in the EPM in Poly(I:C)-treated animals represent light-aversion (American Psychiatric Association, 2022).

3 Single Cell Transcriptomic Analysis of Fetal Brain Reveals Sex Differences in Cellular Responses to Maternal Immune Activation

3.1 Introduction

Immune activation during development is associated with differences in offspring brain development and behavior (see chapter titled "Introduction to the Immune System and Sex as Variables in Brain Development"). Using the maternal immune activation animal model, in which pregnant animals receive immune stimulation via compounds such as Poly(I:C), researchers have sought to better understand the critical time periods, cell types, tissues, and pathways involved in the maternal and fetal response.

As the name of the model suggests, the field has focused predominantly on the activation of the maternal immune system as the effector system leading to the brain and behavior changes in offspring. Important work has uncovered roles for maternal cytokines interleukin (IL)-6 and IL-17a in MIA-associated changes in fetal brain and offspring behavior, and more recently placental IL-6 has also been suggested as a key player in MIA phenotypes (Smith et al., 2007; Choi et al., 2016; Wu et al., 2017). However, determining how these immune signals are detected in the fetal brain and how they go on to impact brain development has not yet been determined. The variability in MIA model paradigms and phenotypes adds complexity to the puzzle, since models may be working through different or overlapping mechanisms (Estes et al., 2019; Kowash et al., 2019).

Microglia have been proposed as a putative cell type which may respond to MIA within the fetal brain and facilitate neurological changes (Bilbo and Stevens, 2017; Prins et al., 2018; Han et al., 2021). Microglia are the resident immune cells in the brain parenchyma. These cells have the capacity to sense TLR3 ligands such as Poly(I:C), respond to cytokines of known relevance to MIA such as IL-6 and IL-17a through their receptors, and also to produce IL-6 (Sarma et al., 2009; Kummer et al., 2021). Microglia have emerged as crucial mediators of normal brain development, with roles in neurogenesis and synaptic pruning (Cunningham et al., 2013; Bilbo and Stevens, 2017). This immune competency paired with neurodevelopmental importance has led researchers to investigate microglia as a promising candidate responder cell population.

Differential gene expression, morphological changes, and motility changes in microglia after MIA have been reported in multiple studies and across species, though the presence of morphological changes is debated (Mattei et al., 2017; Gumusoglu and Stevens, 2019; Ozaki et al., 2020). Gene expression studies found that microglia had a transiently adult-like transcriptome after E14.5 or E12.5 MIA and a long-lived blunted immune response after E9.5 MIA (Matcovitch-Natan et al., 2016; Hayes et al., 2022). Injection of IL-17a into the lateral ventricles of the fetal brain resulted in activation and relocalization of microglia, linking microglial activation to this key MIA cytokine (Sasaki et al., 2020). However, reports of microglial phenotypes after MIA have been somewhat conflicting, with variability depending on stimulus, read-out, age, sex, and brain region (Smolders et al., 2018).

More recently, research groups have investigated another set of brain-resident macrophages called border-associated macrophages (BAM) as possible responders and effectors of MIA-induced signaling. Like microglia, BAM derive from yolk-sac progenitors and have the capacity to sense and produce cytokines, but have distinct developmental trajectories, expression profiles, and localization in the CNS (Goldmann et al., 2016; Utz et al., 2020; Prinz et al., 2021). BAM are located in brain border regions such as the meninges, perivascular space, and choroid plexus.

There are fewer data on the impact of MIA on brain borders and BAM cells, but they suggest brain macrophages respond to MIA. Cui et al. (2020) found that E12.5 Poly(I:C) MIA led to inflammation and increased numbers of phagocytic macrophages in the choroid plexus by E14.5, as well as pro-inflammatory cytokines and chemokines in the cerebrospinal fluid (CSF). Single-cell RNA sequencing from our lab showed an increase in the number of cells and inflammatory gene expression in BAM-like clusters after Poly(I:C) MIA, which was dependent on fetal *Trif* (an adapter for TLR3 signaling) (Nichols et al., 2020). These studies suggest a role for BAM in the fetal MIA response, with the potential to impact on far-reaching brain structures via secretion of immune molecules into the CSF.

Overall, brain myeloid cell responses seem to be a feature of the MIA model, though how important these changes are for neurological phenotypes is yet to be elucidated. How myeloid and/or other fetal brain cell types are responding to acute immune signals after MIA, and how responses differ by sex, is also still unclear. To further investigate the phenotypes of potential brain responder cells after E12.5 Poly(I:C) MIA, we performed single cell RNA sequencing across two time points, 6hr and 24hr after injection, in an attempt to capture any peak in response or difference in immune resolution between groups. We separated samples by sex where it was technically possible in order to investigate sex differences, which have been under-studied in the MIA model (Coiro and Pollak, 2019). To capture a broad population of these brain myeloid cells as well as additional brain cell types which may be responding, we used magnetic-activated cell sorting to enrich for myeloid cells. We used the Parse Biosciences sample collection and library preparation process, based on the SPLiT-seq method (Rosenberg et al., 2018). This method enables fixation and freezing of samples and thereby collection of multiple samples over time, to generate enough replicates for differential gene expression analysis. Sequencing data were processed using Parse Biosciences software to generate a gene-count matrix, which was further processed primarily using BioConductor packages and following guidance from the book, Orchestrating Single Cell Analysis with BioConductor (Huber et al., 2015; Amezquita et al., 2019). The following analyses

demonstrate the unexpected involvement of fetal ependymal cells in the response to MIA, and the presence of sex differences in expression profiles of ependymal and neural progenitor cells after MIA.

3.2 Methods

3.2.1 Animals

All animal housing and experiments were approved by the University of California, Berkeley Animal Care and Use Committee. Animal housing conditions and breeding protocol were the same as listed in the previous chapter, except that pregnant dams remained co-housed with 1-2 other females during pregnancy until time of sacrifice. Wild type mice used for both the maternal immune activation validation and responsiveness study as well as for the single-cell RNA sequencing study were purchased from Charles River Laboratories (C57BL/6NCrl, Strain 027).

3.2.2 Maternal Immune Activation with Poly(I:C)

The MIA induction protocol was the same as described in the previous chapter except a series of Poly(I:C) solutions of different concentrations were prepared for the MIA dosing study, resulting in final doses of 5, 10, or 20mg/kg Poly(I:C). For the single-cell RNA sequencing study, 10mg/kg dose Poly(I:C) was used and dams were sacrificed either 6 or 24 hours after injection. Dams were injected between 10-11:15 am, and when sacrificing multiple litters in a day, injections were staggered by 30 minutes to account for differences in time of sacrifice.

3.2.3 Sample Collection

Dams were euthanized using isofluorane and cervical dislocation, and the uterine horn was dissected and placed in ice-cold PBS. Each fetus was dissected, tails were collected for sex chromosome genotyping, and then individual fetal brains were collected under a dissection microscope. Brain tissue was minced with a blade and collected in individual conical tubes containing 5mL Dulbecco's Modified Eagle Medium. Between each embryo brain dissection, tubes were inverted to expose tissue to the media. Tail DNA preparation for sex chromosome genotyping was performed using the alkaline lysis method detailed in the prior chapter. Tail tissue lysis was performed at 98℃ for 15min, followed by neutralization and 1:40 dilution with water. Sex chromosome genotyping PCR was performed using the 2X KAPA2G Fast HotStart Ready Mix and *Rbm31* primers (Table 3.1), which detect an 84bp deletion in the X-linked version of the gene relative to the Y-linked version (Tunster, 2017). PCR conditions were as follows: 95℃ for 3min, then 35 cycles of 95℃ for 15sec, 60℃ for 15sec, and 72℃ for 5sec, then a final step at 72℃ for 1min. Amplified PCR samples were visualized via agarose gel electrophoresis to genotype each embryo. While PCR and gel were in process, each sample was triturated 20 times using a flame-polished glass

pipette pre-wet with media, and placed on a shaker at low setting in a 4℃ cold room. After getting genotyping results, 2 or more same-sex brain samples were pooled together into 50mL conical tubes (equal numbers of male embryos were pooled and female embryos were pooled). The experiment was aborted if a litter had fewer than 2 female or fewer than 2 male embryos.

Table 3.1: Rbm31 primers used for sex chromosome genotyping of fetal samples

Target gene	Primer	Sequence	Amplicon length	Notes
Rbm31	F	CACCTTAAGAACAAGCCAATACA	X: 269bp Y: 353bp	1 Band = X only 2 Bands = XY
Rbm31	R	GGCTTGTCCTGAAAACATTTGG		

Pooled samples were spun at 200 g at 4°C for 5 minutes to pellet, then re-suspended in 5mL freshly-prepared 37°C digestion buffer (5.2 mL of HBSS (containing Ca2+ and Mg2+), 26 µL of Liberase, 26µL of DNAseI (10U/ul)). Samples were incubated at 37°C with constant rotation for 15 min. At 5 min, 26µl more DNaseI was added if white DNA clumps were visible. At 15 min, any undigested large DNA clumps were removed and 5ml Wash Buffer (HBSS (Ca2+ and Mg2+ free) with 10% FBS) was added to stop the digestion reaction. Samples were then transferred to 15mL conical tubes, spun at 200 g at 4°C for 5 min to pellet. Samples were resuspended in 1mL RBC lysis buffer (Alfa Aesar, #J62150-AK), and incubated at room temperature for 10 min. 10mL Wash Buffer was added to stop the reaction, and cells were spun at 200 g at 4°C for 5 min. Samples were resuspended in a solution of 400µl MACS Buffer (PBS with 0.5% BSA) + 10.25µl Cd11b beads (Miltenyi Biotec, #130-049-601), and incubated at room temperature for 15 min, followed by a wash in 10mL MACS Buffer. Samples were resuspended in 1mL MACS Buffer and strained with a 40µm cell strainer before loading onto MACS MS columns and collecting negative fractions (Miltenyi Biotec, #130-042-201). Columns were washed twice with 1mL and 500µL MACS Buffer before removing columns from the magnet and collecting enriched fractions. Samples were then spun down at 200g at 4°C for 10 min. Cells from enriched fractions were resuspended in 100µl of Cell Buffer from the Parse Biosciences Cell Fixation Kit (#SB1001).

Cells were counted and percent viability estimated using trypan blue (8µl cell susp + 2µl dye) and a hemocytometer under a light microscope. Samples passed quality control with >75% viability and cell number >12,500. An additional 685µl Cell Buffer was added to samples and 25µl of this mix was removed for additional QC using flow cytometry. The remaining sample was processed using the Parse Biosciences Cell Fixation Kit protocol (#SB1001). Fixed samples were frozen at -80°C until library preparation.

3.2.4 Library Preparation and Sequencing

Library preparation and sequencing was performed by the UC Davis DNA Technologies & Expression Analysis Core Laboratory. The Parse Biosciences Single Cell Whole Transcriptome 100k

kit (SKU: SB2001) was used for library preparation. An estimated 17k cells were split over 5 sublibaries (~1000 cells in sublibrary 5 and ~4000 cells in each of the other sublibraries). 100 ng of cDNA was used as input for library prep for all sub libraries, and 10 PCR cycles were used. Libraries were sequenced on a NovaSeq S4 sequencer with 150bp paired-end reads. Sublibrary 5 was sequenced in two pilot runs on two different lanes for a total of 31.6 Gb data (108 million reads), and sublibraries 1-4 were also each sequenced in separate lanes for 261 Gb of total data (867 million reads). This resulted in just under 1 billion total reads for the experiment. All runs had >86% of bases >= Q30, with a mean quality score range 34.51-34.73.

3.2.5 Genome Alignment and Generating a Counts Matrix

The Berkeley Research Computing group High Performance Computing clusters were used for initial genome alignment and demultiplexing of barcoded sequences. First, the four FASTQ files generated from sublibrary 5, consisting of read 1 and read 2 files from two different sequencing runs, were concatenated using the `cat` command in the terminal. We combined sequences from read 1 or read 2 for each sequencing run, resulting in two FASTQ files.

We used the Parse Biosciences' software pipeline (version 0.9.6p) for processing of FASTQ sequence files. The mm10 mouse genome was used for alignment. The `wget` function was used to download the genome file `Mus_musculus.GRCm38.93.gtf.gz`. Next we used the `split-pipe` function from Parse Biosciences' software for each sublibrary to align sequences to the genome and generate count matrices. Sample names were specified in the call to split-pipe, so this metadata was assigned to individual cells in the output. To combine the sublibraries, we again called `split-pipe` under combine mode (`--mode comb`) and specified that unfiltered data should be returned.

3.2.6 Analysis Pipeline Development and Execution in R

Unfiltered count matrix data were processed and analysed using the R programming language (v4.2.1) and associated open source packages, which will be cited in the following sections (R Core Team, 2022).

The analysis pipeline was created using code and recommendations from several sources, including the Parse Biosciences' support suite, Seurat package, and primarily the Orchestrating Single-Cell Analysis with Bioconductor book (Amezquita et al., 2019). Resources informing the differential gene analysis include guides from Law et al. and the EdgeR package User's Guide Chen et al. (2008). Data was primarily stored in the SingleCellExperiment object class for analysis, with the use of the tidySingleCellExperiment package to allow tidyverse-style manipulation of the data (Amezquita et al., 2019; Mangiola, 2022).

Data were imported into RStudio (v2022.12.0) for processing, statistical analysis, and visualization (RStudio Team, 2022). The core tidyverse packages (v1.3.2) and the here package (v1.0.1) were used to import, clean, and process data (Wickham et al., 2019; Müller, 2020). For packages available in

Bioconductor, version 3.16 packages were used in analysis. Tables and plots were generated using the knitr (v1.40), kableExtra (v1.3.4), gt (v0.8.0), scales (v1.2.1), and ggplot2 packages Wickham et al. (2019). For packages available in Bioconductor, version 3.16 packages were used in analysis.

statistical analyses were performed using the R stats (v4.2.1), limma (v3.54.0), multcomp (1.4.20), broom (v1.0.1), and rstatix (0.7.1) packages [@limma; @multcomp; @broom; @rstatix].

3.2.7 Cell Filtering

An appropriate transcript count threshold for cell filtering was determined using the `barcodeRanks` function from the DropletUtils package (Lun et al., 2019). Because sublibraries had different sequencing depth and samples also varied in transcript count, thresholds were determined separately for each set of cells within a given sublibrary-sample pair (e.g. sublibrary1_sampleAA). Barcode rank was calculated according to transcript count and the inflection point in the associated curve was determined, with a lower bound set using `lowest_UMI = 400`. Plots were inspected manually and thresholds reassigned for 4 of the 175 sublibrary-sample groups in which thresholds were computationally called at too high a level. After these inflection thresholds were applied, 19303 cells remained. Percent mitochondrial gene counts as well as total transcript counts and total genes detected were calculated for each cell using `perCellQCMetrics` from the scuttle package (McCarthy et al., 2017). A threshold for high mitochondrial content was calculated using the `isOutlier` function, also from scuttle, using `nmads = 2` to mark as outliers cells more than 2 median absolute deviations from the median (110 cells discarded). A threshold for high transcript count was similarly calculated using a less stringent threshold of `nmads = 4` (728 cells discarded).

3.2.8 Chromosomal Sex Estimation

Counts for all Y chromosome genes expressed in the data set after cell filtering were summed for each cell using the `perCellQCMetrics` function (McCarthy et al., 2017). Y gene names were ascertained using the biomaRt package (Durinck et al., 2005). To determine appropriate thresholds of Y gene or Xist expression to use for calling estimated chromosomal sex, thresholds were applied to the samples with known sex (E13.5 samples) and metrics were selected to correctly label the highest number of cells. Due to the sparsity of the data, sex could not be estimated for a proportion of cells, which were excluded from comparisons between female and male samples.

3.2.9 Gene Filtering

Genes were discarded that were not detected in the cells remaining after cell filtering. Detectable was defined as genes with >1 UMI in at least 2 cells. 38141 genes were discarded and after filtering, 16091 genes remained in the data set.

3.2.10 Normalization and Batch Correction

Size factors were calculated for each cell using `computeSumFactors` from the scran package (Lun et al., 2016). These size factors account for differences in library size (before computing the factors, cells were clustered using `quickCluster` so cells with similar transcriptomes are grouped). Using these size factors, counts were then normalized and log transformed using `logNormCounts` from the scuttle package (McCarthy et al., 2017). The mean-variance relationship across genes was modeled using `modelGeneVar` with sublibrary-sample groups as a blocking factor (Lun et al., 2016). The top 20% highly variable genes were calculated using `getTopHVGs` (1384 genes) and then used as input for principal component analysis via `runPCA`. (Lun et al., 2016; McCarthy et al., 2017). PCA values were then used as input for the Harmony batch correction function `HarmonyMatrix` (Korsunsky et al., 2022). Variables input for correction included sublibrary, sample, and whether or not the sample was concentrated by centrifugation before library prep. The Harmony algorithm went through 12 iterations before converging, and the resulting embeddings were then added as reduced dimensions to the SingleCellExperiment object. tSNE visualization was performed before and after Harmony batch correction to inspect the effectiveness of the correction, using the PCs from PCA for the before visualization (Maaten and Hinton, 2008; McCarthy et al., 2017). UMAP visualization was also performed for comparison (McCarthy et al., 2017; McInnes et al., 2018).

3.2.11 Clustering and Cell Type Identification

The louvain method of the `clusterCells` function from the scran package was used to cluster cells, using the Harmony embeddings and k = 25 (Lun et al., 2016). Multiple values for k were tested to obtain a resolution differentiating main cell types (e.g. neural, microglia, BAM) without multiple subclusters. This resulted in 13 clusters. Clusters were assigned cell type classification using a combination of techniques: 1) a list of marker genes was generated from the data, 2) expression of marker genes from the literature were plotted, and 3) cell transcriptomes were compared to reference data sets with cell type labels to calculate similarity scores. For the first approach, the `scoreMarkers` function from the scran package was used to generate a list of marker genes by cluster and the top five genes from each cluster with the highest area under the curve were plotted. For the second approach, expression of canonical myeloid, microglial and border associate macrophage (BAM) gene markers were plotted to assign several clusters to these cell type identities. In the third approach, additional cell types were determined by comparing to reference data sets using the SingleR package (Aran et al., 2019). The pre-packaged mouse RNAseq reference from Benayoun et al. (2019) from the celldex package was used to assign ependymal, endothelial, neuron, and NPC clusters (Aran et al., 2019). These assignments were confirmed by comparing the marker genes from these clusters to publicly available and searchable sequencing data sets including those from Cao et al. (2019), La Manno et al. (2021), and Loo et al. (2019). Additionally, clusters 9, 10, 11, and 12 were compared to developmental microglia and BAM transcriptomes from Utz et al. (2020) using SingleR.

3.2.12 Differential Expression Analysis

Differential expression was measured by summing counts within a cell-type/sample to create pseudobulk samples and applying a generalized linear model and quasi-likelihood F-tests via the edgeR package (Chen et al., 2016). Cells with unknown sex were excluded, and then counts were summed using the `aggregateAcrossCells` function from the scuttle package (McCarthy et al., 2017). Cell-type/sample groups with fewer than three cells were excluded and then cell types with less than three replicates for any of the eight experimental groups were excluded from further analysis.

The following analysis was performed for each cell type. Genes with reasonably consistent expression across samples were retained for analysis using edgeR's `filterByExpr` function (Chen et al., 2016). Normalization, estimation of dispersion and applying of quasi-likelihood F-tests were then performed using edgeR's `calcNormFactors`, `estimateDisp`, `glmQLFit` and `glmQLFTest` methods. The design matrix used for the linear model was generated using `model.matrix(~0 + group)` where group represents each sample, split by sex for E12.5 samples (R Core Team, 2022). Specific contrasts (experimental group comparisons) of interest were generated using the `makeContrasts` function from the limma package, specified in Table 3.2 (Ritchie et al., 2015). Differentially expressed genes with a Benjamini Hochberg adjusted p-value less than 0.1 were reported. Y chromosome genes as well as *Xist* and *Tsix* were filtered out of the results for V_12_MvsF and V_13_MvsF contrasts.

3.2.13 Gene Set Analysis

The `enrichGO` function from the clusterProfiler package was used to assess differentially expressed gene sets for enrichment of gene ontology pathways (Yu et al., 2012). Gene sets for each cell type and contrast with adjusted p-values less than 0.1 were investigated. Gene ontology results with Benjamini Hochberg adjusted p-values less than 0.05 and including more than one input gene were reported.

3.3 Results

3.3.1 Poly(I:C) Injection at E12.5 Causes Transient Sickness Phenotypes in Dams and Some Litter Loss

For both MIA validation and the single-cell RNA sequencing experiment, C57BL/6 dams were bred with C57BL/6 sires and presence of a plug marked embryonic day (E) 0.5. Dams were weighed on E10.5 or E11.5 to confirm pregnancy, and then treated with Poly(I:C) or vehicle via intraperitoneal injection on E12.5. A cohort of dams were injected with 20mg/kg Poly(I:C), a dose commonly used in the literature, and followed during pregnancy to observe sickness phenotypes and pregnancy outcomes.

Table 3.2: Contrasts used to compare coefficients in the differential expression linear model. Coefficients estimate the expression level for each group. The research question motivating a given contrast is listed, as well as the abbreviation for the contrast used in reporting differentially expressed genes for that contrast. The mathematical formula comparing the coefficients to generate each contrast is also listed.

Research Question	Contrast	Comparisons Between Coefficients
Effect of Poly(I:C) in E12.5 Females	F_12_PvsV	E12.5 PolyI:C Female - E12.5 Veh Female
Effect of Poly(I:C) in E12.5 Males	M_12_PvsV	E12.5 PolyI:C Male - E12.5 Veh Male
Effect of Poly(I:C) in E13.5 Females	F_13_PvsV	E13.5 PolyI:C Female - E13.5 Veh Female
Effect of Poly(I:C) in E13.5 Males	M_13_PvsV	E13.5 PolyI:C Male - E13.5 Veh Male
Effect of Sex at E12.5 in Veh-treated	V_12_MvsF	E12.5 Veh Male - E12.5 Veh Female
Effect of Sex at E13.5 in Veh-treated	V_13_MvsF	E13.5 Veh Male - E13.5 Veh Female
Differential effect of Poly(I:C) in Males vs Females at E12.5	Tx_12_MvsF	(E12.5 PolyI:C Male - E12.5 Veh Male) - (E12.5 PolyI:C Female - E12.5 Veh Female)
Differential effect of Poly(I:C) in Males vs Females at E13.5	Tx_13_MvsF	(E13.5 PolyI:C Male - E13.5 Veh Male) - (E13.5 PolyI:C Female - E13.5 Veh Female)
Effect of Poly(I:C) in all groups combined	Tx	(E12.5 PolyI:C Female + E12.5 PolyI:C Male + E13.5 PolyI:C Female + E13.5 PolyI:C Male) - (E12.5 Veh Female + E12.5 Veh Male + E13.5 Veh Female + E13.5 Veh Male)

3.3.1.1 Altered Pregnancy Outcomes and Weight Loss in Poly(I:C)-Treated Dams

Of the 8 dams treated with 20mg/kg Poly(I:C), 2 had pregnancy loss following injection and 2 lost litters on the day of birth (dead and/or cannibalized pups), while the 10 dams injected with vehicle had successful litters (Figure 3.1). Fisher's exact test was used to determine if there was a significant association between pregnancy outcomes and treatment. There was a significant association between these two variables (p = 0.0229).

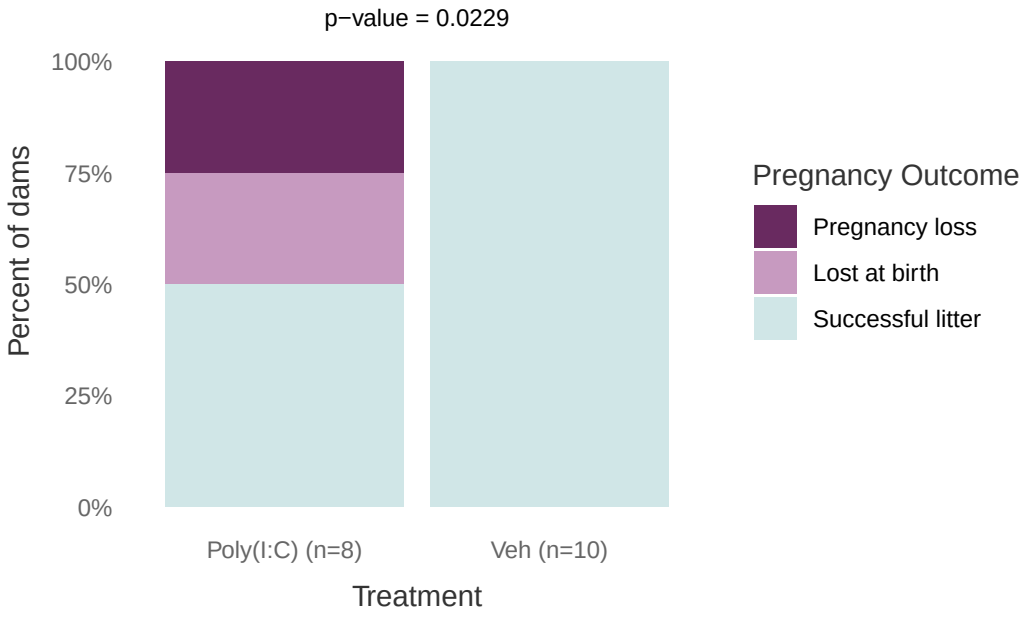


Figure 3.1: Pregnancy outcomes of mice treated with 20mg/kg Poly(I:C) or vehicle at E12.5

At 3 and 6 hours after injection, dam weights were similar to or below their initial weight measured at the time of injection for both vehicle and Poly(I:C) groups (Figure 3.2). The effect of Poly(I:C) treatment on dam weight was evident at 24hr, at which point vehicle-treated dams regained and surpassed their initial weight, while Poly(I:C)-treated dams generally remained below their initial weight from the prior day. At 24 hours, the weight of 8 Poly(I:C)-treated dams (mean = 96.3% of initial, sd = 3.4%) relative to 10 vehicle-treated dams (mean = 103.1% of initial, sd = 1.1%), was significantly lower ($t(8.2) = 5.4$, $p = 0.00031$). By 48 hours, most dams had surpassed their initial weight, though two Poly(I:C)-treated dams had weights lower than their initial weight, indicating pregnancy loss.

To determine whether a lower dose could prevent pregnancy loss while still producing an immune response, we treated additional dams with lower doses of Poly(I:C), either 10mg/kg or 5mg/kg, and tracked their weight and sickness phenotypes. Though sample sizes for this dose study were small, no significant differences in weight were observed at 24 or 48 hours compared to vehicle, indicating less toxicity and pregnancy loss than the 20mg/kg dose (Figure 3.3).

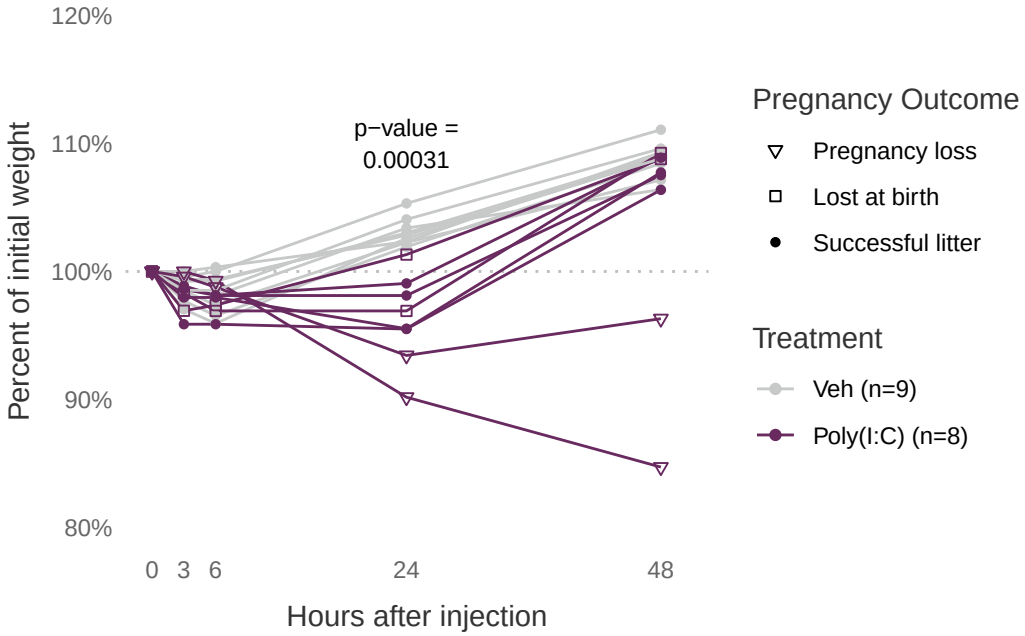


Figure 3.2: Dam weight outcomes for the two days post injection of 20mg/kg Poly(I:C) or vehicle at E12.5. Ultimate pregnancy outcomes are plotted by different point shapes, and treatment by line and point color. Weights are shown as a percentage of initial weight at time of injection.

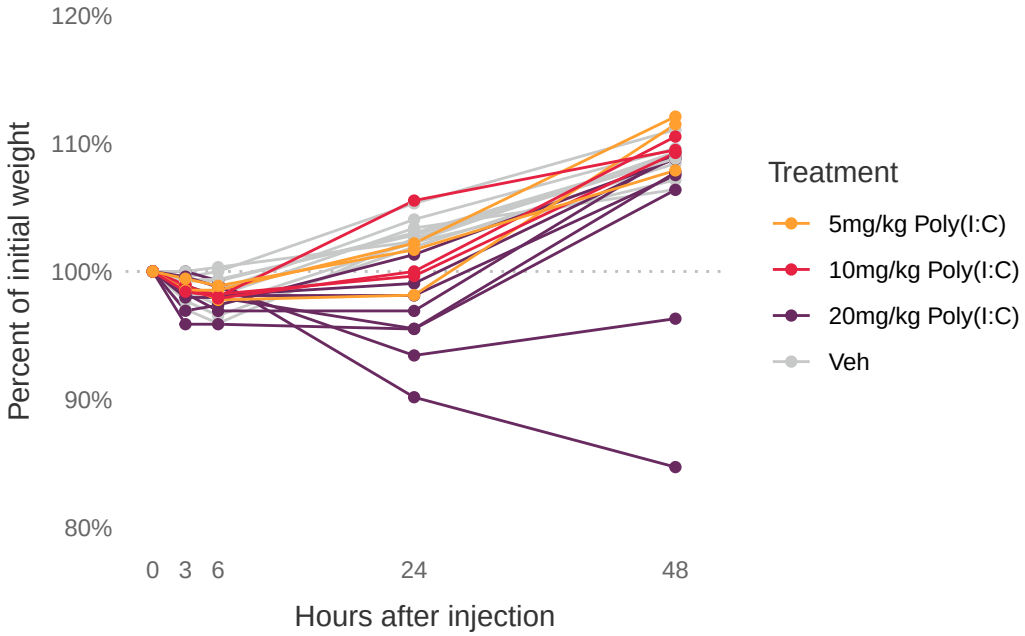


Figure 3.3: Dam weight outcomes of a Poly(I:C) dosing study. 5, 10, or 20 mg/kg Poly(I:C) or vehicle were injected at E12.5 and weights taken over the following two days.

3.3.1.2 Sickness Appearance and Behaviors

We observed a series of sickness phenotypes in treated dams and measured their severity using a 3 point scale (0 = none, 1 = moderate, 2 = severe). The phenotypes we investigated were determined based observation, and included scruffy coat, ptosis, piloerection, labored breathing, divots along the sides of the back, curled body posture, activity prior to handling, and activity when handled. The majority of dams from each Poly(I:C) treatment group demonstrated at least one sickness phenotype in the acute phase of immune activation 3 hours after injection (Figure 3.6). Of all 18 Poly(I:C)-treated dams, 14 dams displayed 1 or more sickness phenotypes and 10 dams displayed 2 or more at 3 hours. In contrast, in the vehicle-treated group, 2 out of 12 dams displayed sickness phenotypes at 3 hours.

To get a better sense of the overall impact of treatment on sickness phenotypes across time, we combined data from all sickness phenotypes into a composite sickness score, where higher values represent more types of phenotypes observed and/or higher severity of phenotypes. These composite scores were plotted over time for each treatment and dose, demonstrating an overall sickness burden at 3 hr after injection across all doses of Poly(I:C) (Figure 3.4). Two mice from the 20mg/kg Poly(I:C) treatment group still had moderate or severe phenotypes at 24 hours. All mice returned to baseline scores for the phenotypes tested by 48 hours.

A linear regression was used to test if Poly(I:C) dose significantly predicted composite sickness score at 3 hours. The fitted linear model was: sickness score = 0.07937*dose + 0.41827, with

dose in units of mg/kg (Figure 3.5). The overall regression was statistically significant, and dose significantly predicted sickness score (R^2 = 0.2701, p = 0.002733).

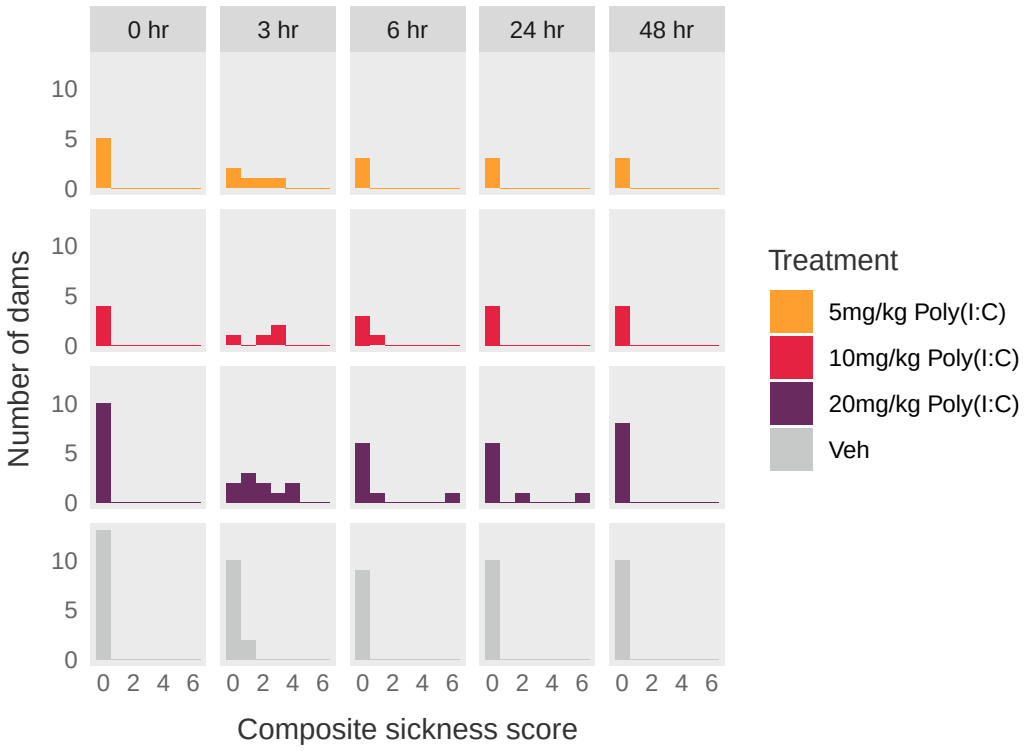


Figure 3.4: Composite sickness scores over time for dams injected with vehicle or different doses of Poly(I:C) at E12.5.

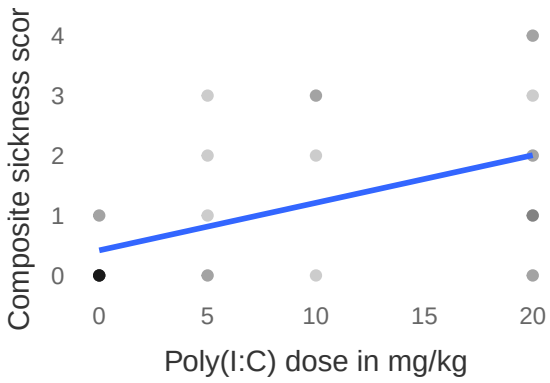


Figure 3.5: Composite sickeness scores from 3 hours after injection are plotted according to Poly(I:C) dose in mg/kg. The linear regression line is shown.

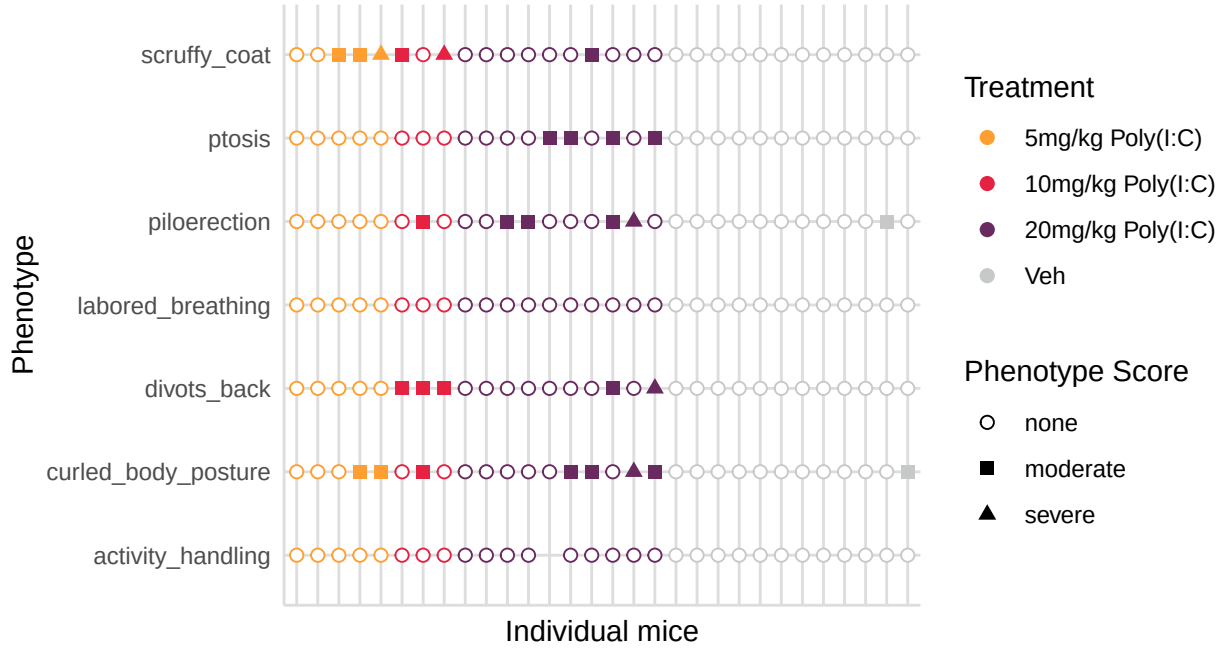


Figure 3.6: Sickness phenotypes for individual dams 3 hours after treatment with vehicle or different doses of Poly(I:C). Phenotypes were scored on a 3 point scale (none, moderate, severe). Each verticle line in the chart represents the observed phenotypes for an individual mouse.

3.3.2 Embryo Brain Samples Exposed to MIA were Collected for Single-Cell RNA Sequencing

Embryo brain samples were collected from litters 6 hours (E12.5) or 24 hours (E13.5) after injection. Veh and Poly(I:C) treated litters were paired and sacrificed on the same day when possible (14 litters), though some litters don't have a paired litter if dams were not pregnant or there weren't enough embryos of a given sex to continue the experiment for that litter (8 litters). At the E12.5 time point, female and male samples were pooled in an equal ratio to generate enough cells for downstream processing. The collected samples are listed in Table 3.3, with overall sample number n per group as listed in Table 3.4.

3.3.3 Cell and Gene Quality Control

Single cell sequencing data were processed via the Parse Biosciences software pipeline to align to the mouse genome, demultiplex barcodes, and generate a raw count matrix. Barcodes with low transcript counts were filtered out of the data set, likely representing ambient RNA or broken,

Table 3.3: Samples collected and sequenced for single cell RNA sequencing

Sample Name	Litter	Treatment	Embryonic Day	Sample Sex	Collection Day
C_BOTH	C	Poly(I:C)	12.5	BOTH	1
G_BOTH	G	Veh	12.5	BOTH	2
H_BOTH	H	Poly(I:C)	12.5	BOTH	2
I_BOTH	I	Poly(I:C)	12.5	BOTH	3
J_BOTH	J	Veh	12.5	BOTH	4
K_BOTH	K	Veh	12.5	BOTH	5
L_BOTH	L	Poly(I:C)	12.5	BOTH	5
M_FEMALE	M	Veh	13.5	FEMALE	6
M_MALE	M	Veh	13.5	MALE	6
N_FEMALE	N	Poly(I:C)	13.5	FEMALE	6
N_MALE	N	Poly(I:C)	13.5	MALE	6
P_BOTH	P	Veh	12.5	BOTH	7
Q_BOTH	Q	Veh	12.5	BOTH	8
R_BOTH	R	Poly(I:C)	12.5	BOTH	8
U_FEMALE	U	Poly(I:C)	13.5	FEMALE	9
U_MALE	U	Poly(I:C)	13.5	MALE	9
W_FEMALE	W	Poly(I:C)	13.5	FEMALE	10
W_MALE	W	Poly(I:C)	13.5	MALE	10
AA_FEMALE	AA	Veh	13.5	FEMALE	11
AA_MALE	AA	Veh	13.5	MALE	11
BB_FEMALE	BB	Veh	13.5	FEMALE	12
BB_MALE	BB	Veh	13.5	MALE	12
CC_FEMALE	CC	Poly(I:C)	13.5	FEMALE	12
CC_MALE	CC	Poly(I:C)	13.5	MALE	12
FF_BOTH	FF	Veh	12.5	BOTH	13
GG_BOTH	GG	Poly(I:C)	12.5	BOTH	13
HH_BOTH	HH	Veh	12.5	BOTH	14
II_BOTH	II	Poly(I:C)	12.5	BOTH	14
KK_FEMALE	KK	Veh	13.5	FEMALE	15
KK_MALE	KK	Veh	13.5	MALE	15

Table 3.4: Number of samples collected per group (timepoint, treatment, and sample sex).

Embryonic Day	Treatment	Sample Sex	n
12.5	Veh	BOTH	7
12.5	Poly(I:C)	BOTH	7
13.5	Veh	FEMALE	4
13.5	Veh	MALE	4
13.5	Poly(I:C)	FEMALE	4
13.5	Poly(I:C)	MALE	4

partially sequenced cells (Figure 3.7). Low transcript count filtering was done on a per sublibrary-sample basis to account for baseline differences in read depth, and filtering thresholds were determined by calculating the inflection point of barcode rank plots (Figure 3.8). Filtering on low transcript count discarded 1,441,082 and kept 19,303 barcodes.

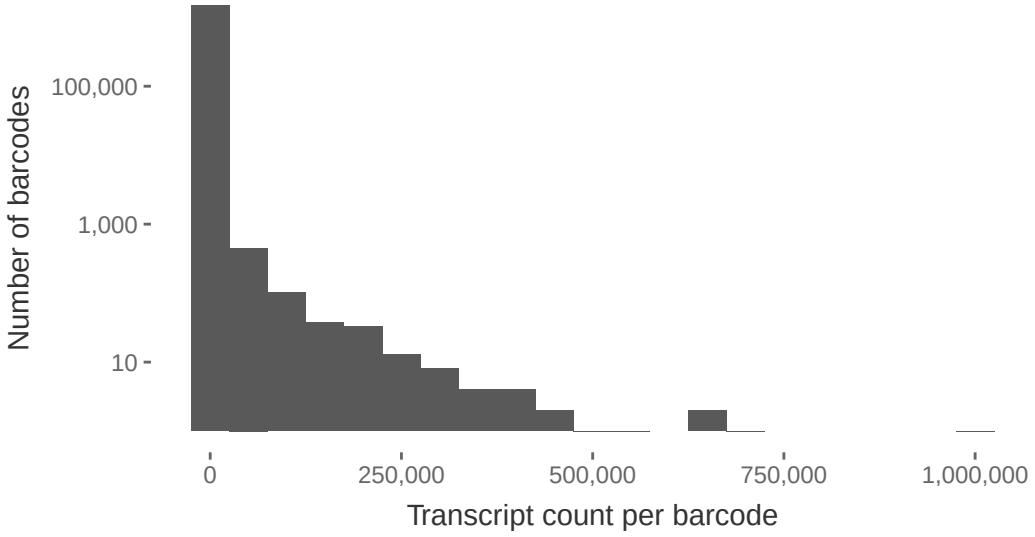


Figure 3.7: Pre-filtering distribution of barcodes with given number of transcript counts from raw data.

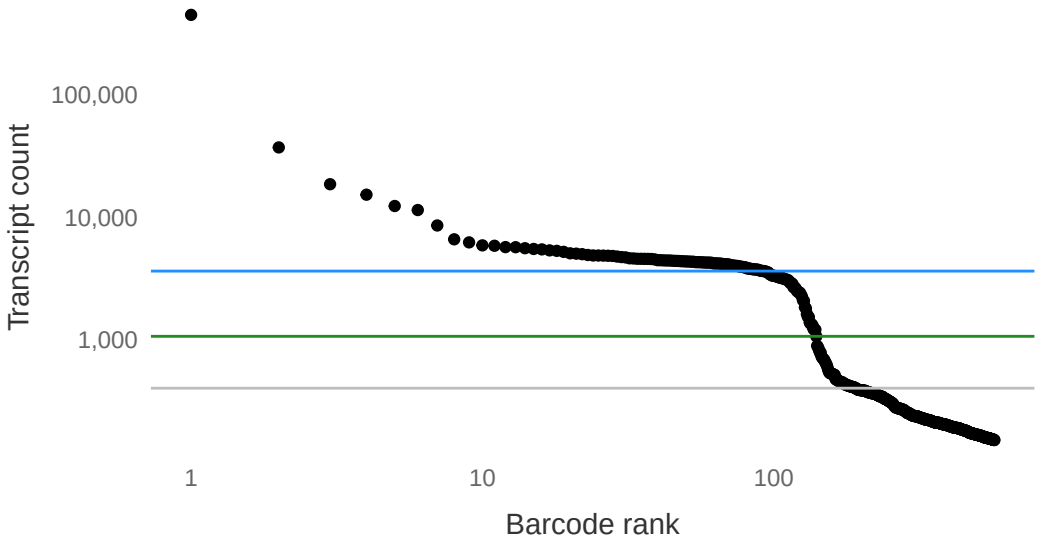


Figure 3.8: An example barcode rank plot used to filter cells. The middle green line marks the inflection point and the top blue line marks the knee point calculated by the barcodeRanks function. The lowest grey line marks the lower bound UMI count parameter for choice of inflection and knee points.

Library quality was good as measured by mitochondrial transcripts, a common quality control metric. Percent of counts coming from mitochondrial transcripts was low overall and generally consistent across samples (Figure 3.9, Figure 3.10). Only 110 cells were discarded as outliers with mitochondrial percent above 6.9%.

To detect possible multiplets, cells were also filtered for transcription count 4 median absolute deviations above the median for their sublibrary-sample group. 728 cells were discarded as potential multiplets, leaving a final count of 18,465 cells in the data set (Figure 3.9, Figure 3.10).

A final filtering step was also applied to remove undetected genes, keeping 16091 features detected in at least two cells.

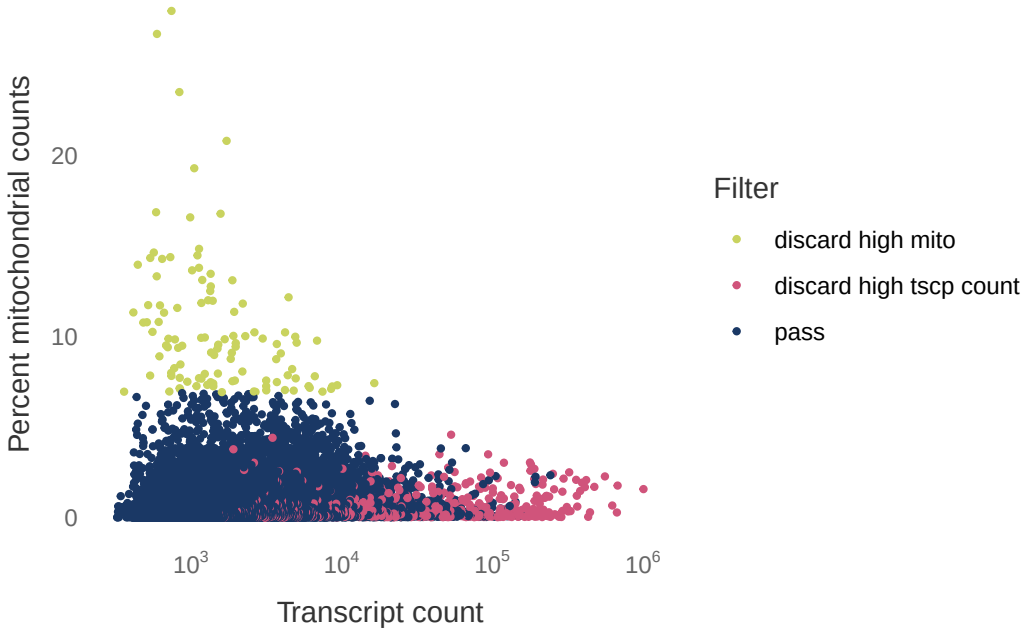


Figure 3.9: Cells were filtered based on a transcript count more than 4 median absolute deviations (MAD) above the median for that cells' sublibrary and sample. Cells with mitochondrial transcript percent 2 MAD above the median for all cells were also filtered out.

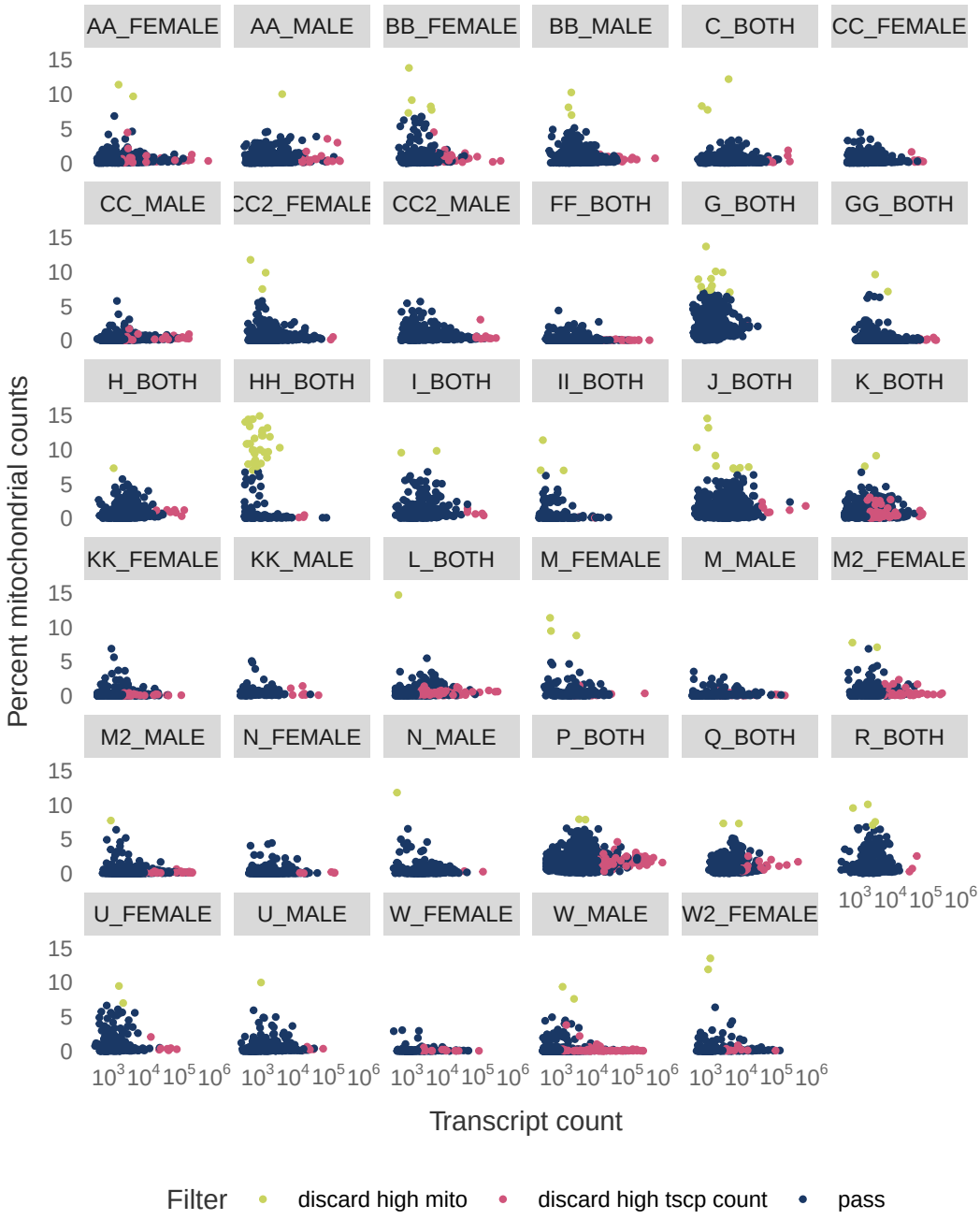


Figure 3.10: Cells were filtered based on a transcript count more than 4 median absolute deviations above the median for that cells' sublibrary and sample. Cells with mitochondrial transcript percent 2 MAD above the median for all cells were also filtered out. Each sub-plot shows a sample containing cells from the five different sublibraries.

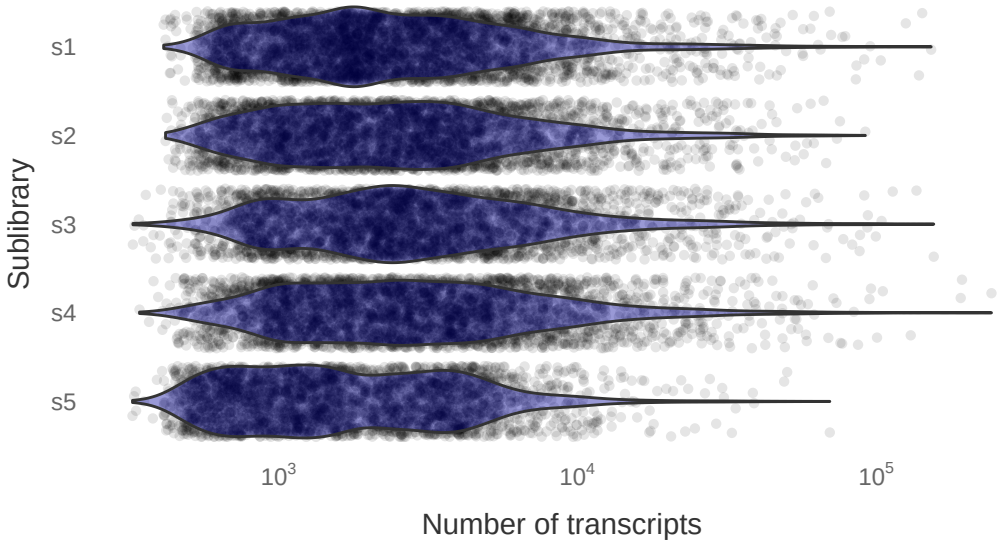


Figure 3.11: Sublibraries varied slightly in sequencing depth, but had generally similar distributions of transcript counts. Individual cells are plotted in the background, overlayed by a blue violin plot showing the overall distribution. Plots show cells remaining after cell filtering.

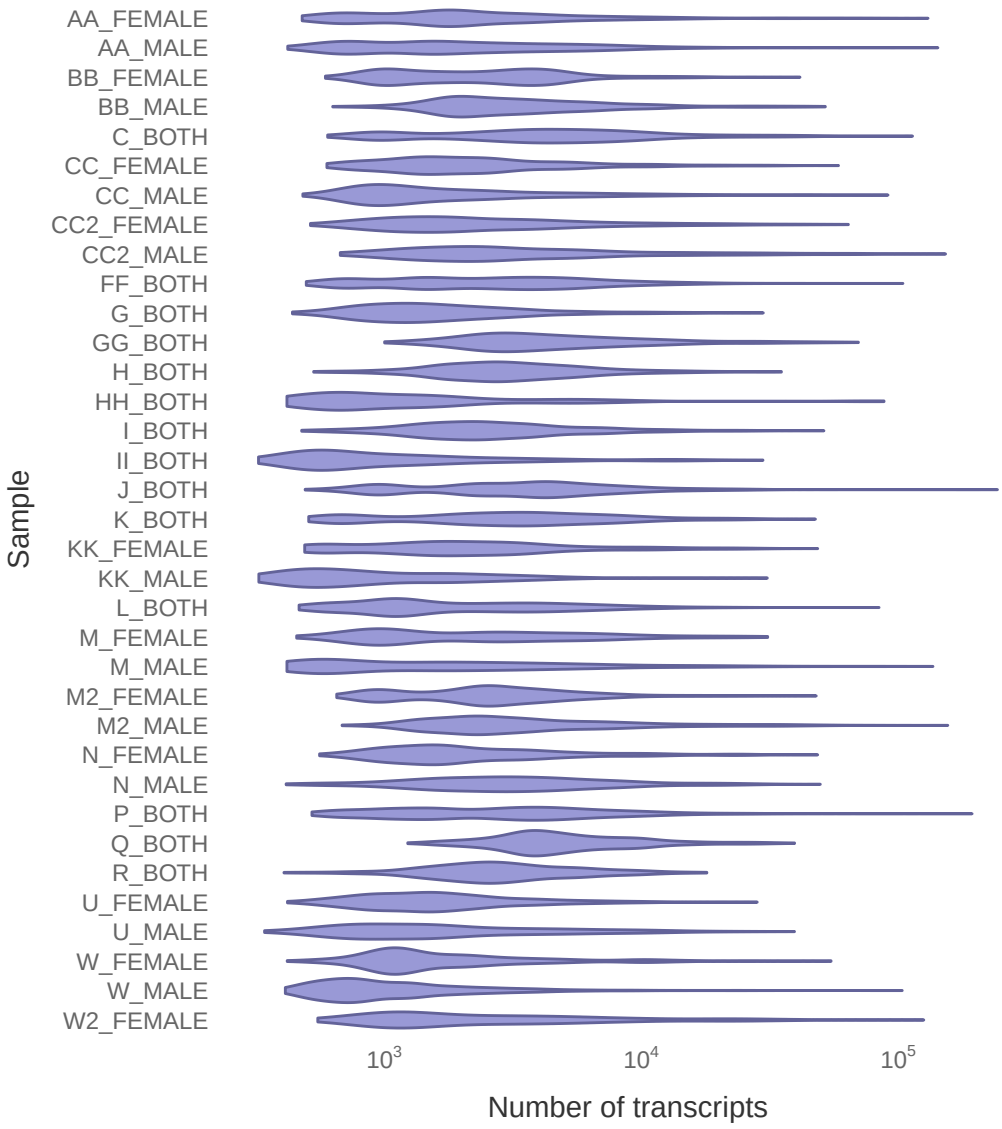
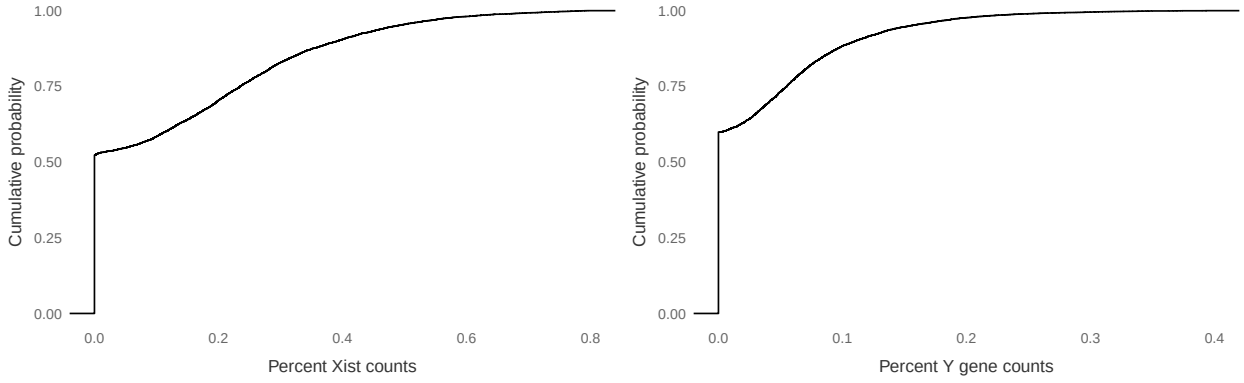


Figure 3.12: Samples showed variation in the number of transcripts per cell. The distribution of transcript counts for each sample is shown in a series of violin plots. Plots show cells remaining after cell filtering.

3.3.4 Bioinformatic Estimates of Sex Chromosome Genotypes

For samples collected at E13.5, the *Rbm31* genotype of each embryo was assessed. This gene is present on the X chromosome and has a Y chromosome gametolog with an 84bp difference in length, which enables PCR genotyping of *Rbm31* to serve as a proxy for chromosomal sex (Tunster, 2017). For E12.5 samples, brains from *Rbm31x+Rbm31y-* female and *Rbm31x+Rbm31y+* male embryos were mixed in equal proportions, so the chromosomal sex of individual cells in those samples is unknown, but can be estimated bioinformatically. To do this, all Y genes in the data set

and the X chromosome gene *Xist* (known to be expressed highly in individuals with more than one X chromosome) were used to assign putative chromosomal sex. Due to the sparsity of single cell data, sex could not be estimated for all cells. Cumulative density plots across the entire data set show the proportion of cells by percent of *Xist* counts and percent of Y genes (Figure 3.13). Thresholds for sex-specific gene expression were applied to the data to assign estimated chromosomal sex. To assess the efficacy of these thresholds, estimated sex was compared to *Rbm31y* genotype for samples with known *Rbm31y* genotype. Figure 3.14 and Figure 3.15 show the distribution of sex-specific gene expression among cells from these samples, and Figure 3.16 shows the overall cell count for each estimated chromosomal genotype grouped by known *Rbm31y* genotype, summarized in Table 3.5.



(a) Xist

(b) Y genes

Figure 3.13: Cumulative density plot showing the proportion of cells expressing the percent of counts from sex-specific genes out of the cell's total counts

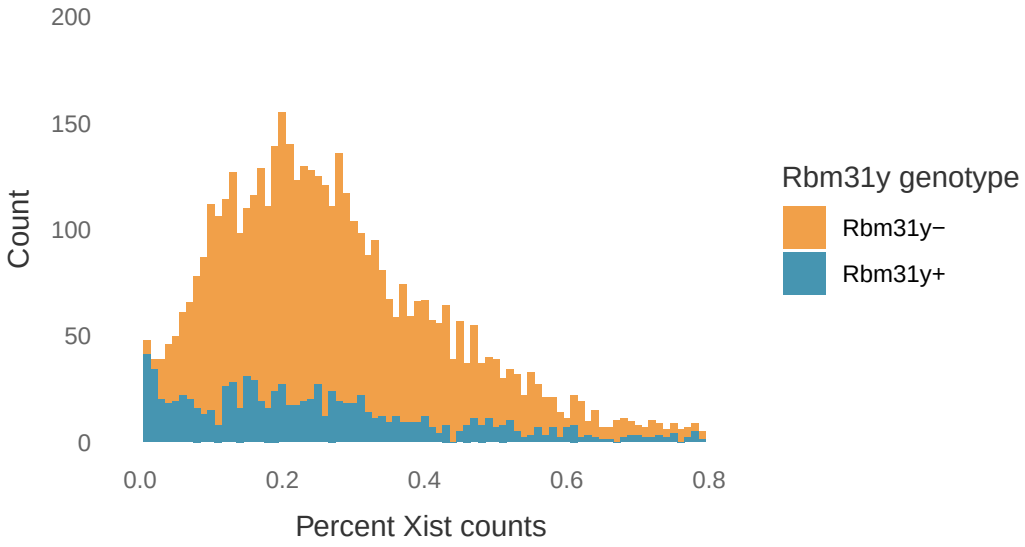


Figure 3.14: Distribution of cells expressing a given percent of Xist gene counts out of the cell's total transcript counts.

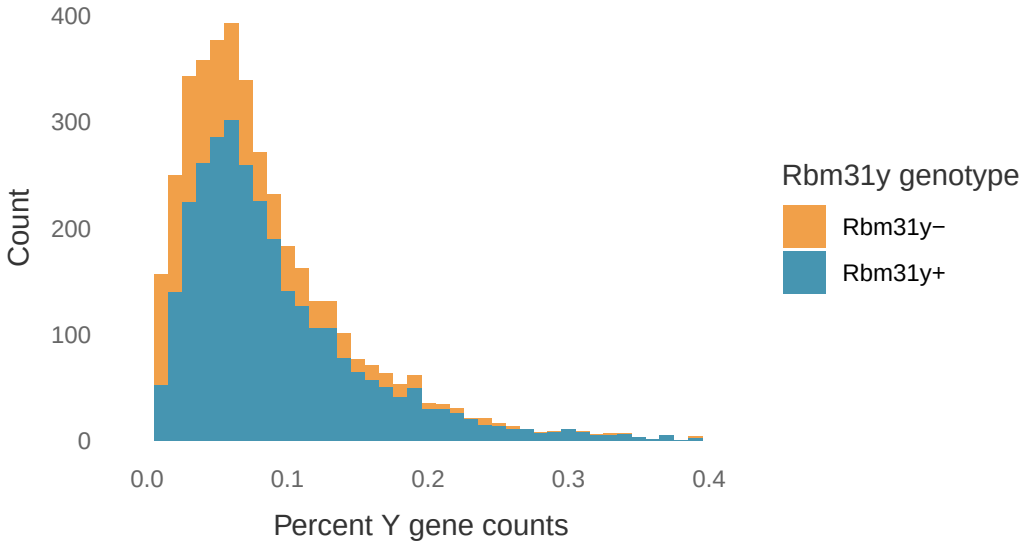


Figure 3.15: Distribution of cells expressing a given percent of Y gene counts out of the cell's total transcript counts.

Table 3.5: Number of cells of each Rbm31y genotype and bioinformatically estimated chromosomal sex. Embryos were genotyped for the Y chromosome gene Rbm31y during sample collection. Embryo brains were either mixed with brains from littermates of the same Rbm31 genotype or a 1:1 ratio of Rbm31y-:Rbm31y+ ("1:1 mixed"). After sequencing, cells were bioinformatically assigned a putative chromosomal sex (XX, XY, or unknown). Percent of total cells in each Rbm31 genotype group (rows) is shown in parentheses.

Rbm31y genotype	XX	XY	unknown
1:1 mixed	4558 (54%)	2415 (29%)	1395 (17%)
Rbm31y-	3826 (82%)	358 (8%)	499 (11%)
Rbm31y+	833 (15%)	2768 (51%)	1813 (33%)

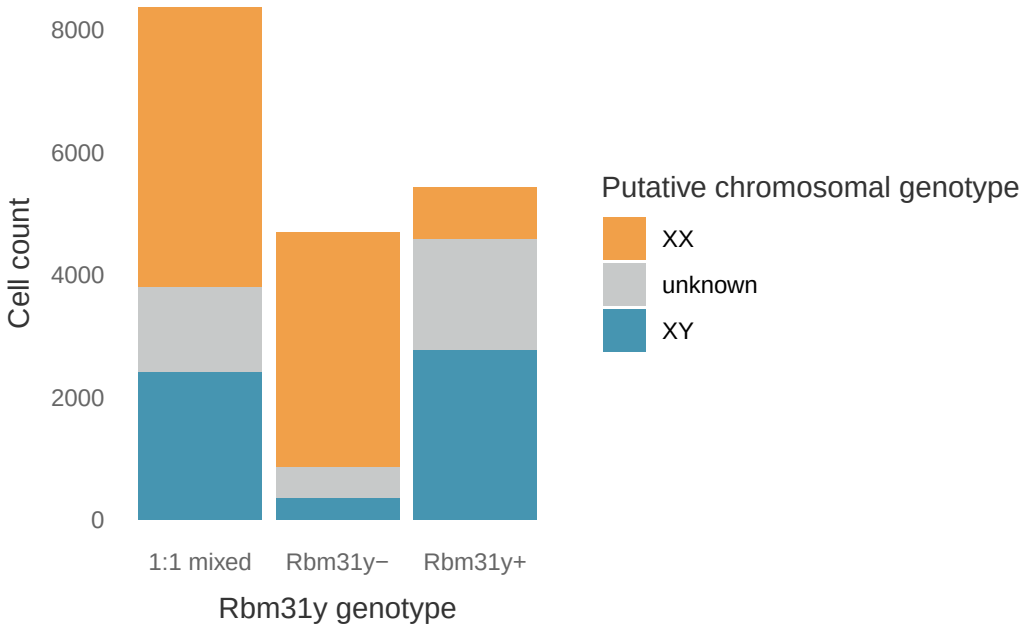


Figure 3.16: Number of cells of each Rbm31 genotype and bioinformatically estimated putative chromosomal sex. Embryos were genotyped for the Y chromosome gene Rbm31y during sample collection. Embryo brains were either mixed with brains from littermates of the same Rbm31y genotype or a 1:1 ratio of Rbm31y-:Rbm31y+ ("1:1 mixed"). After sequencing, cells were bioinformatically assigned a putative chromosomal sex (XX, orange; XY, blue; or unknown, grey).

After cell and gene filtering, 18,465 cells were retained, and of these, 17,070 were assigned an estimated sex chromosome genotype. For cells from E13.5 samples, which had known *Rbm31* genotypes, this information was used to assign estimated sex genotype. For E12.5 samples, sex genotype was estimated bioinformatically as described above using sex chromosome gene count

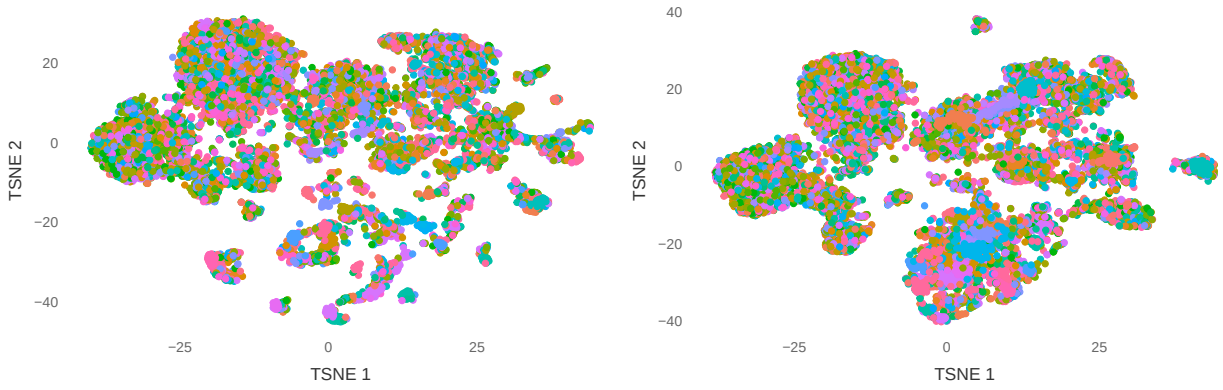
data. Cells with estimated XX genotype will be referred to as female (F) and cells with estimated XY genotype referred to as male (M) in the following text.

Table 3.6: Number of cells by experimental group after filtering.

Embryonic Day	Treatment	Sex (estimated)	n
12	Veh	F	3013
12	Veh	M	1333
12	Veh	unknown	867
12	Poly(I:C)	F	1545
12	Poly(I:C)	M	1082
12	Poly(I:C)	unknown	528
13	Veh	F	2230
13	Veh	M	2786
13	Poly(I:C)	F	2453
13	Poly(I:C)	M	2628

3.3.5 Samples and Experimental Factors were Distributed Across Clusters

After filtering, data was normalized and batch corrected using Harmony to improve cluster identification (Korsunsky et al., 2022). Batch correction successfully grouped individual clusters from different samples into larger overlapping clusters, as can be seen when comparing tSNE visualization before and after batch correction (Figure 3.17).



(a) Before batch correction

(b) After batch correction

Figure 3.17: tSNE visualization of cell clusters before and after batch correction with Harmony.

After batch correction, clustering using the louvain method was performed to group similar cell types or states. Clusters were visualized by both tSNE (Figure 3.18) and UMAP (Figure 3.19) to

view the data in multiple low dimensional representations and take advantage of each method's advantages to view local and global structure. The data were grouped into 13 clusters, including 4-5 larger groups of clusters and 3 smaller clusters which appeared more distinct.

Overall, samples were distributed across clusters, with fairly even distributions of cells in each cluster when split by sex, treatment, or collection time point (Figure 3.20, Figure 3.21, Figure 3.22). Clusters showed some differences in overall transcript count (Figure 3.23).

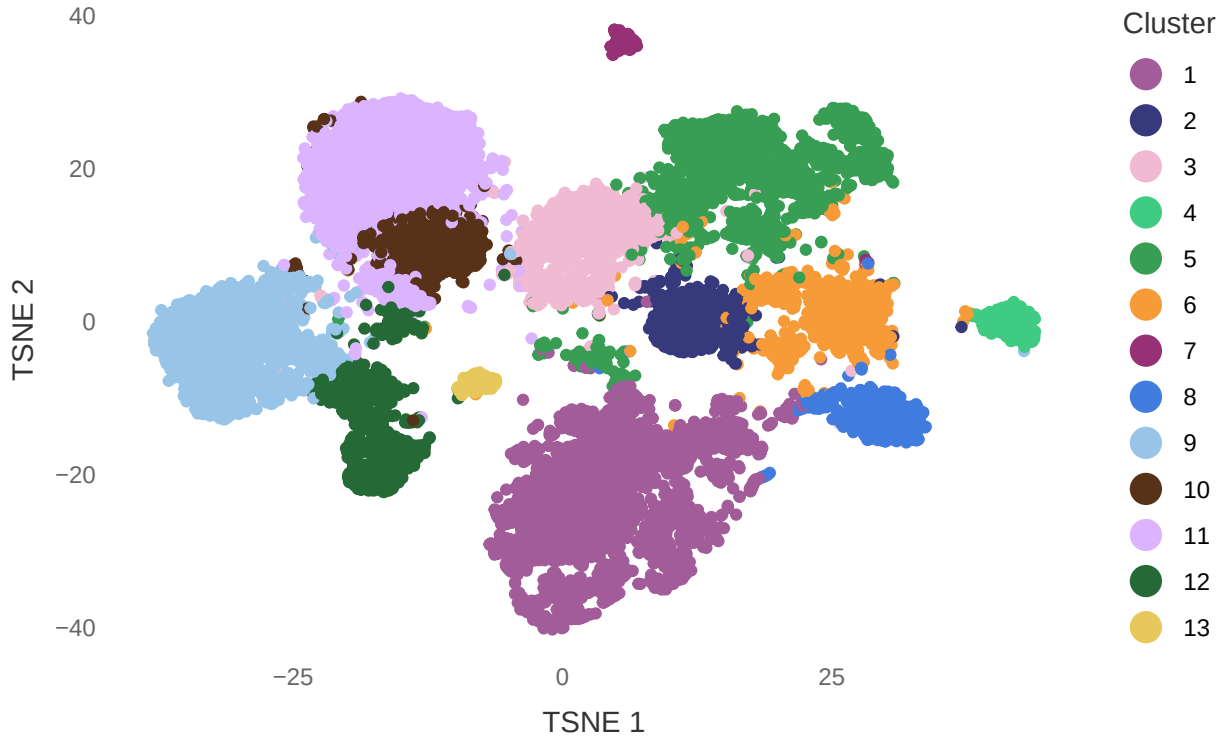


Figure 3.18: tSNE visualization of single-cell RNA expression with cells colored by cluster.

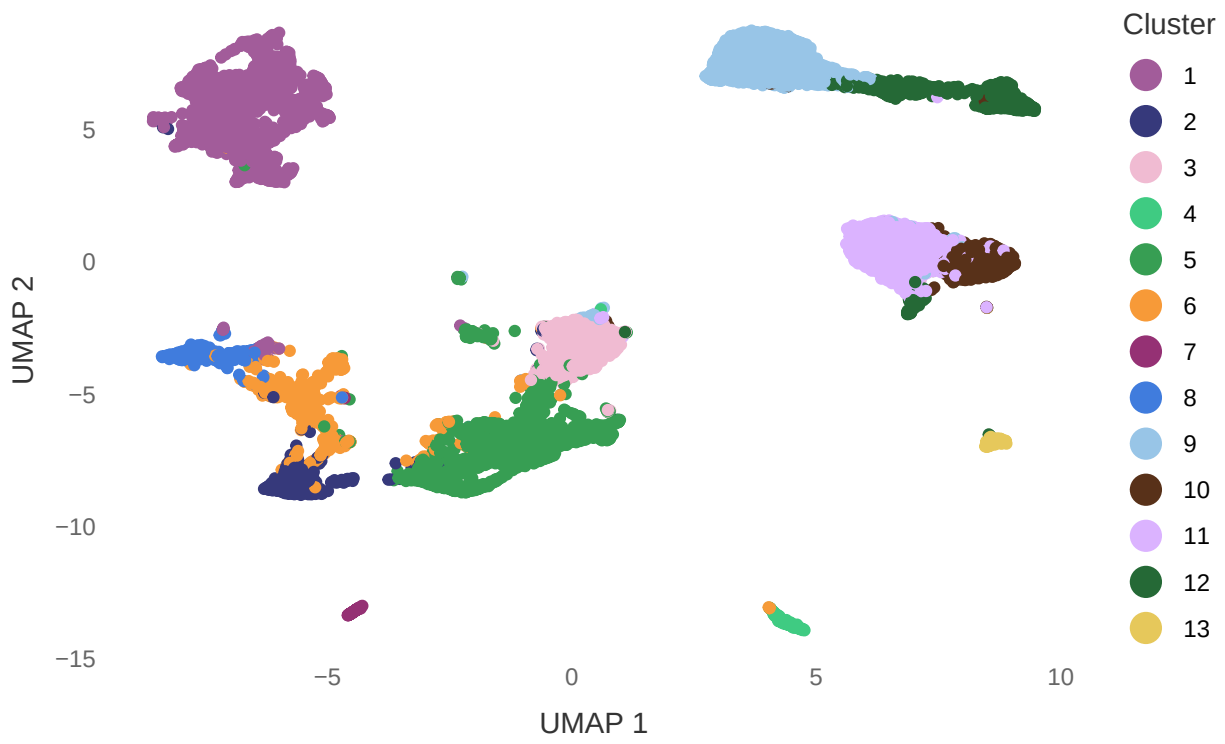
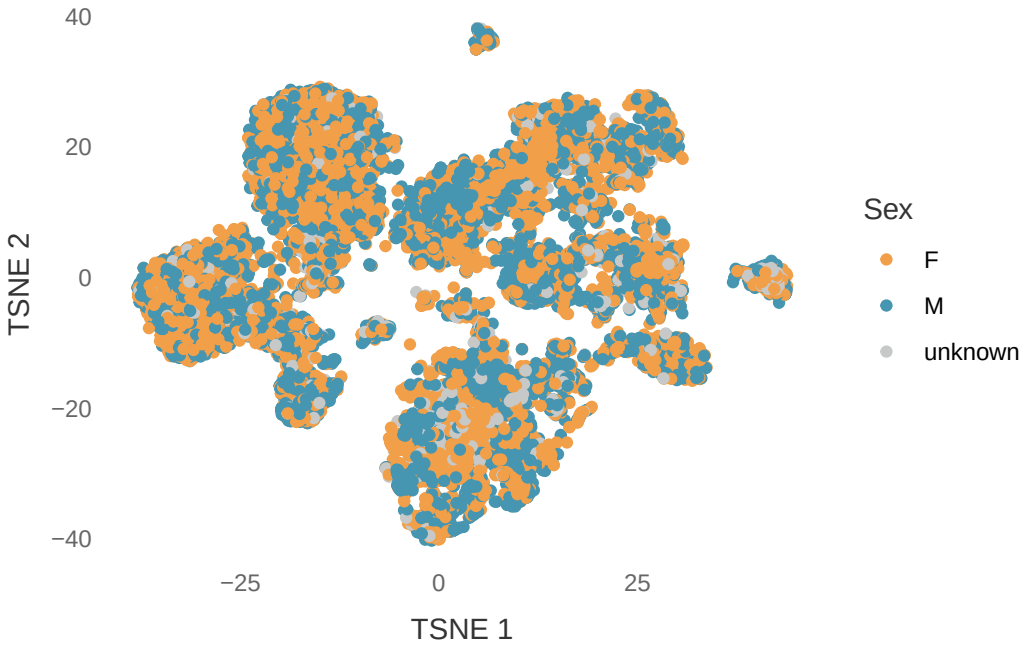
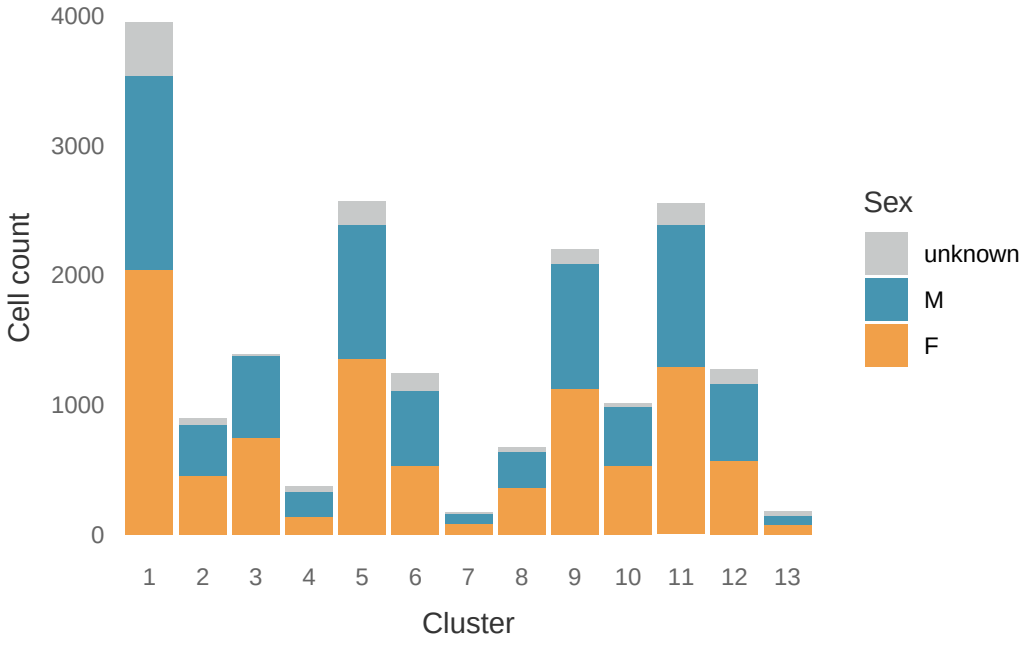


Figure 3.19: UMAP visualization of single-cell RNA expression with cells colored by cluster.

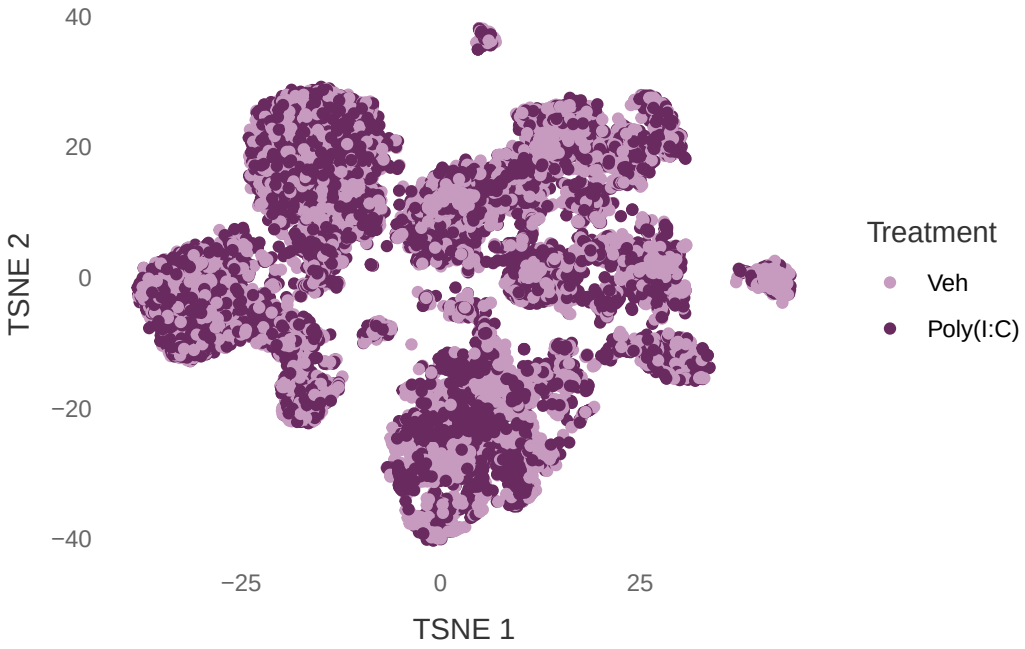


(a) Sex distribution of cells visualized on a t-SNE plot

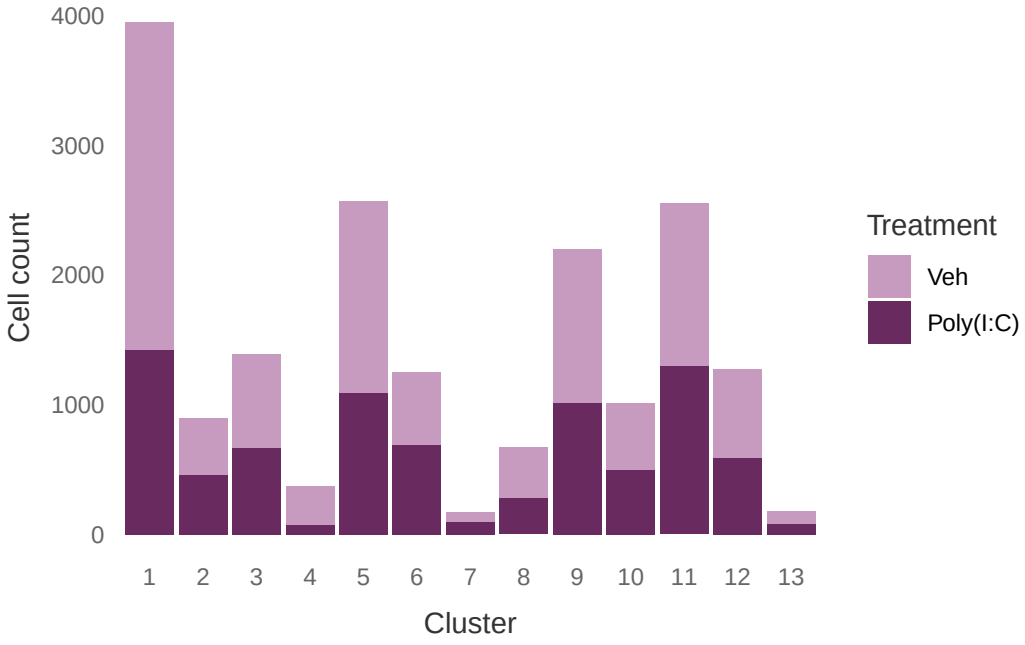


(b) Sex distribution of cells by cluster

Figure 3.20: Sex distribution across clusters in the data set.

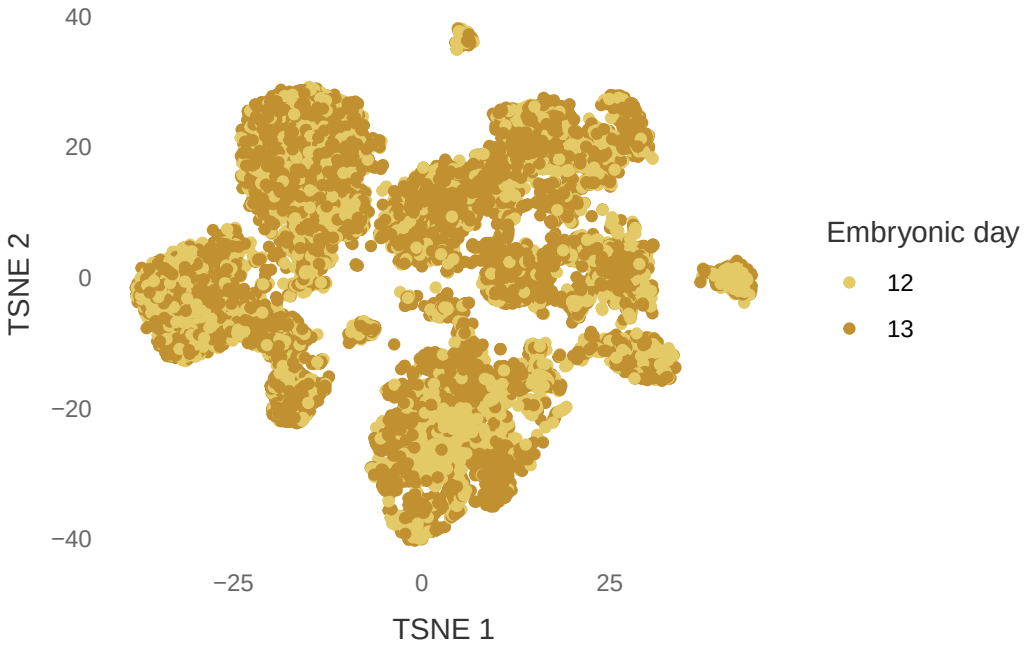


(a) Treatment distribution of cells visualized on a t-SNE plot

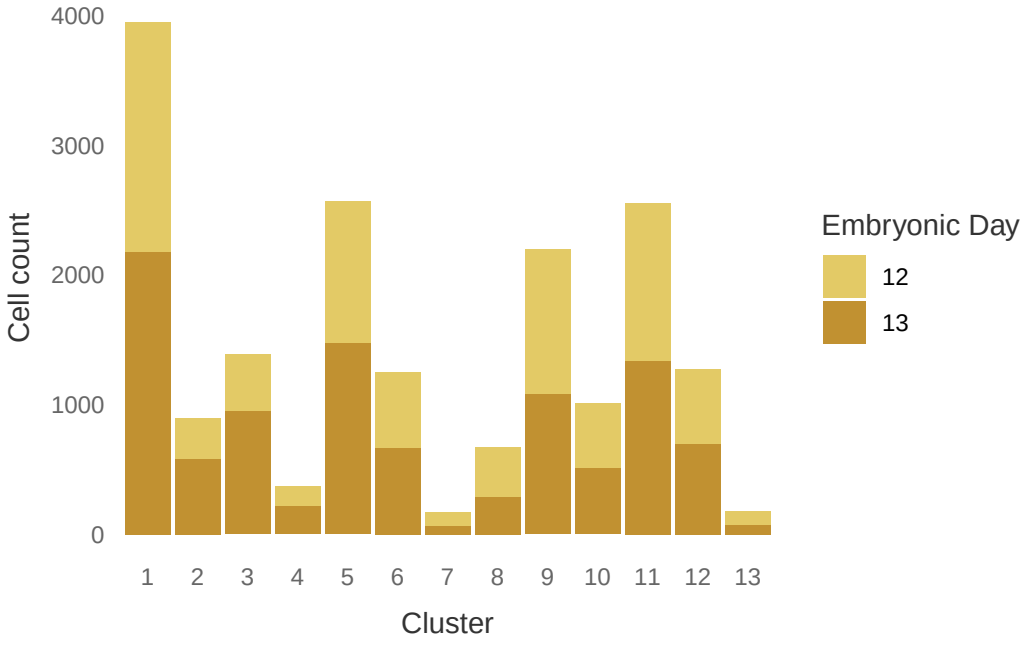


(b) Treatment distribution of cells by cluster

Figure 3.21: Treatment distribution across clusters in the data set.



(a) Age distribution of cells visualized on a t-SNE plot



(b) Age distribution of cells by cluster

Figure 3.22: Age distribution across clusters in the data set.

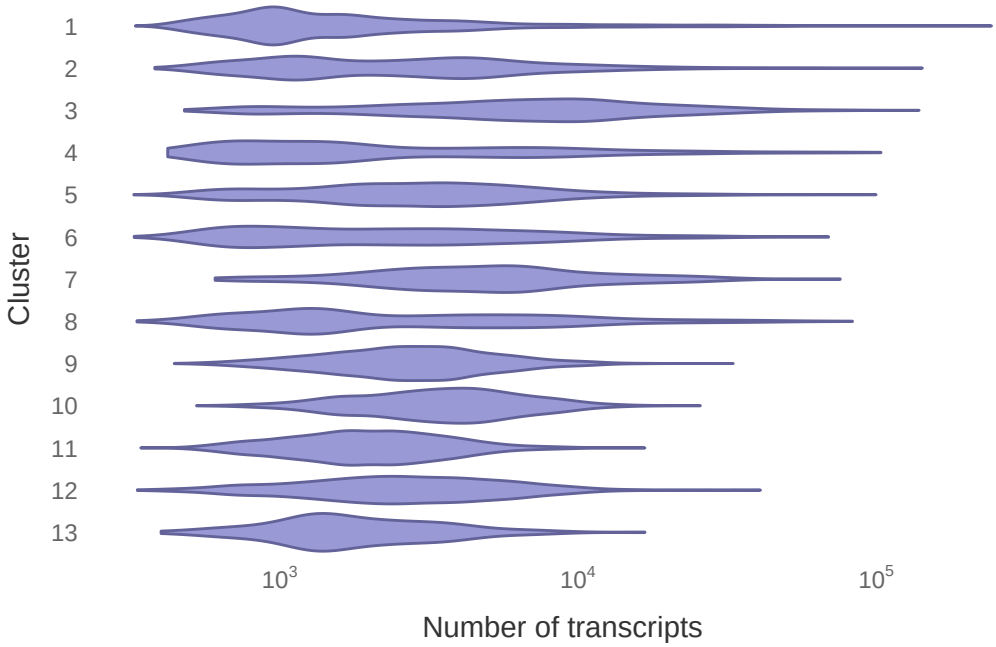


Figure 3.23: Transcript count in cells in each cluster.

3.3.6 Marker Genes and Comparison to Reference Data Sets Identified Microglial, BAM, Ependymal, and Neural Cell Clusters

Cell type identities were investigated by examining genes uniquely highly expressed in particularly clusters. Marker genes were identified using the `scoreMarkers` function, which compares average fold change expression between clusters. We compiled the five genes from each cluster with the highest area under the curve (probability that gene expression in a cell from one cluster will be higher than a cell drawn from all other clusters). These marker genes are shown in Figure 3.24, where lighter colors represent higher average $\log_2$(Fold Change) expression within a cluster, and larger dot size indicates a higher percentage of cells within that cluster express the gene.

Marker genes were searched in published mouse data sets with gene search capabilities to get an initial assessment of cell types represented by each cluster. Data sets examined include Cao et al. (2019), which profiled whole embryos from E9.5-E13.5, La Manno et al. (2021), which profiled mouse brain from E7-E18, and Loo et al. (2019), which profiled cerebral cortex at E14.5 and birth. Based on these gene marker searches, some cluster identities were estimated as follows: 1 - ependymal/choroid plexus; 2 - radial glia/glioblast; 4 - endothelial; 5 - neural progenitors; 7 - notochord/floor plate; 8 - neuroblast/radial glia/isthmic organizer cells; 9, 10, 11, 12 - microglia/macrophages; 13 - neutrophils.

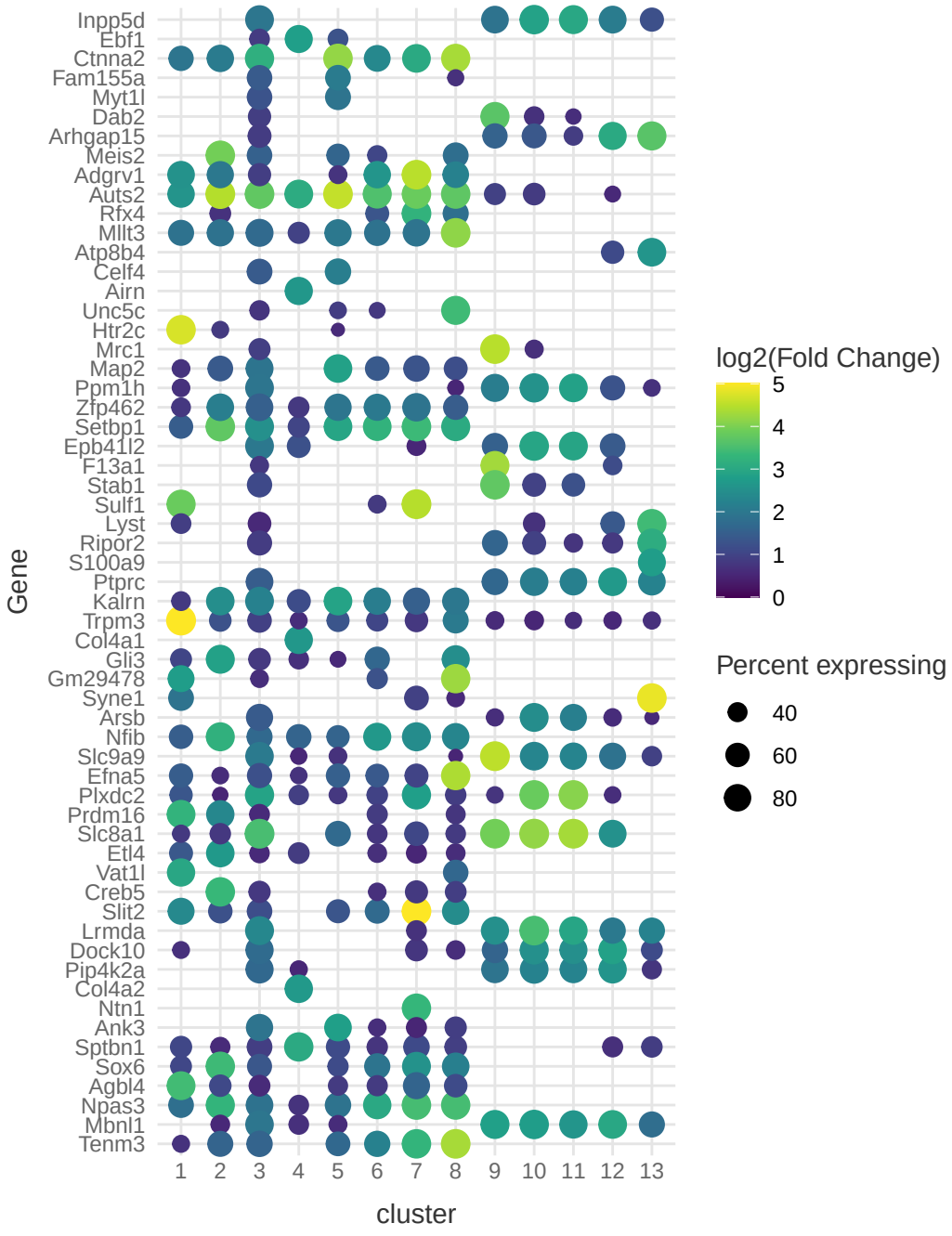


Figure 3.24: Expression of the top five marker genes of each cluster are displayed across clusters. Each dot's color represents the average log fold change for that gene in a cluster. The size of the dot represents the percent of a given cluster expressing that gene.

Assignment of cell types to clusters was further facilitated by comparing cell transcriptomes to published reference data sets. For an initial analysis, a mouse RNAseq data set from Benayoun et al. (2019), available prepackaged through the celldex package, was used as a reference. Cells in

our data set were compared to the transcriptional profiles of labeled cell types in the reference and the resulting similarity scores plotted in a cell-level heatmap (Figure 3.25). A summary heatmap shows the assignment of test cells to reference labels, where brighter squares indicate a greater frequency of cells being assigned a given cell-type label (Figure 3.26). These data largely support the analysis from examining only the top marker genes, suggesting a large microglia-like subset (clusters 9-12) as well as cells with ependymal (1), endothelial (4), granulocyte (13), and neural (clusters 2, 5, 6, 7, 8) signatures.

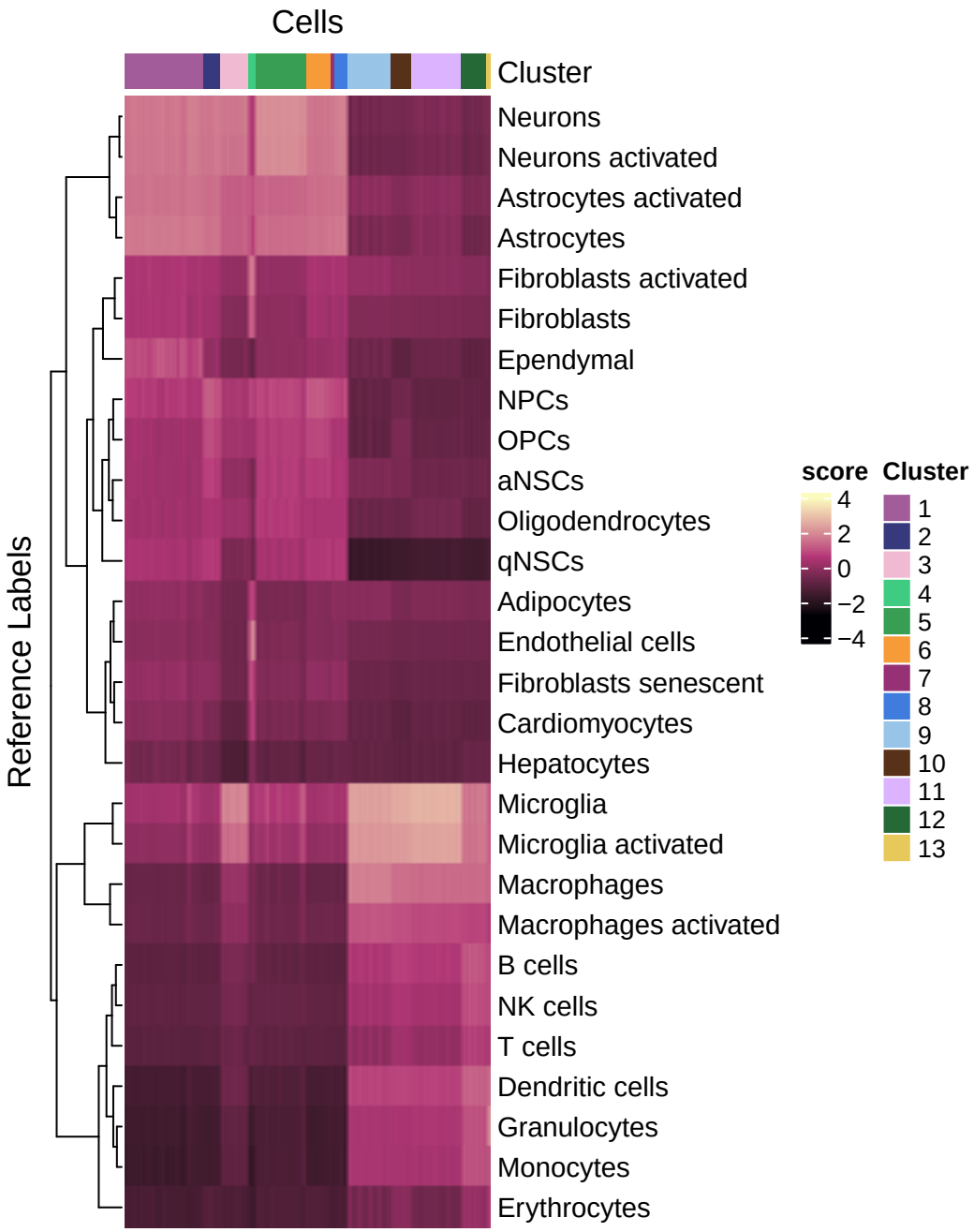


Figure 3.25: Heatmap showing the the similarity score for each cell compared to a given reference cell type label from the Benayoun et al. 2019 data set.

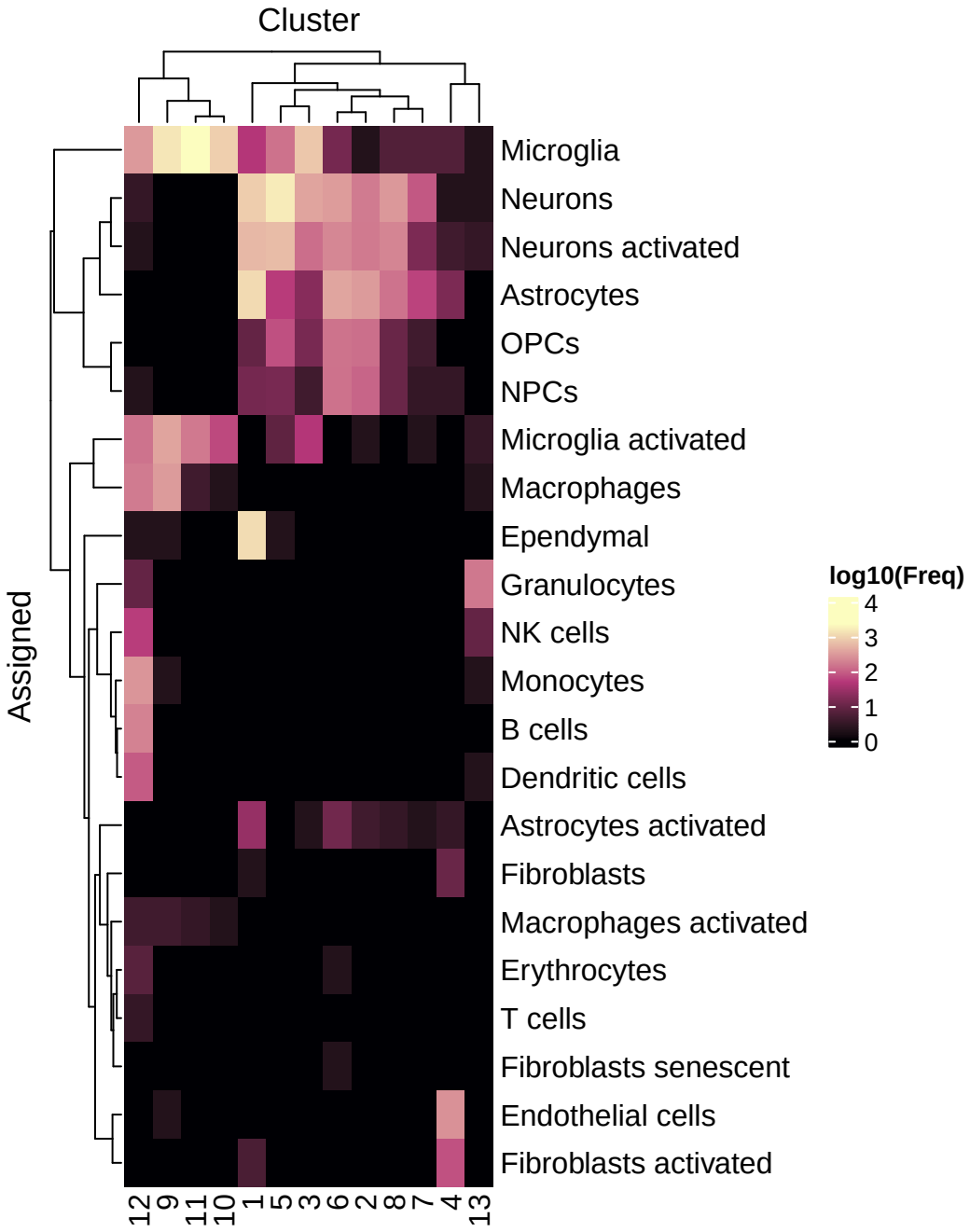


Figure 3.26: Heatmap showing the frequency of cells from a given cluster being assigned to a given reference cell type label from the Benayoun et al. 2019 data set.

To get additional insight into myeloid subsets at developmental time points, the same SingleR similarity score procedure was applied to cells in myeloid clusters (9, 10, 11, 12) compared to a reference data set from Utz et al. (2020), which contains samples of microglia and BAM from E10.5-E18.5. This comparison shows cluster 9 with higher similarity to reference BAM samples,

clusters 10 and 11 with similarity to microglia at multiple ages, and cluster 12 with most similarity to early E10.5 microglia (Figure 3.27, Figure 3.28).

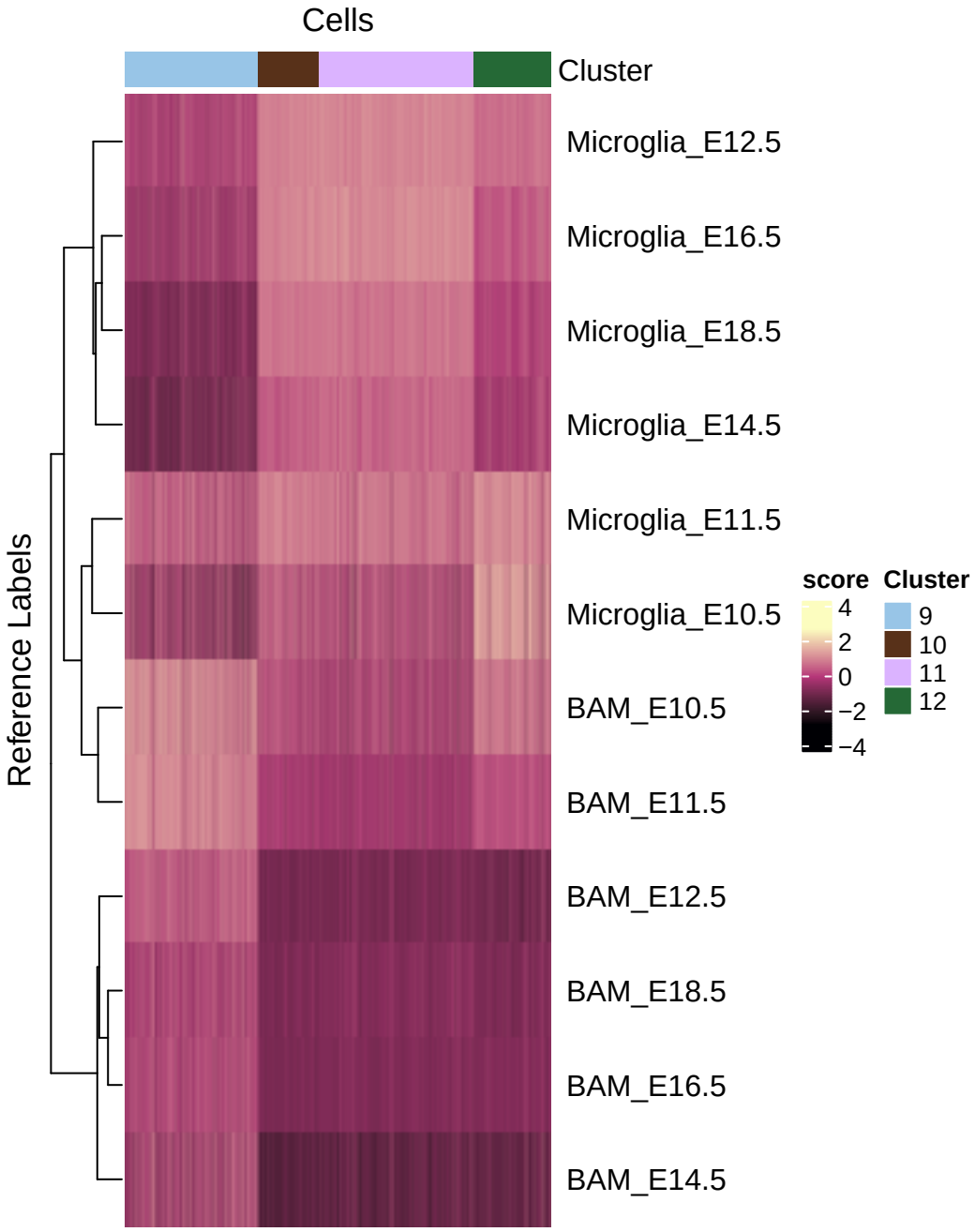


Figure 3.27: Heatmap showing the the similarity score for each cell compared to a given reference cell type label from the Utz et al. 2020 data set.

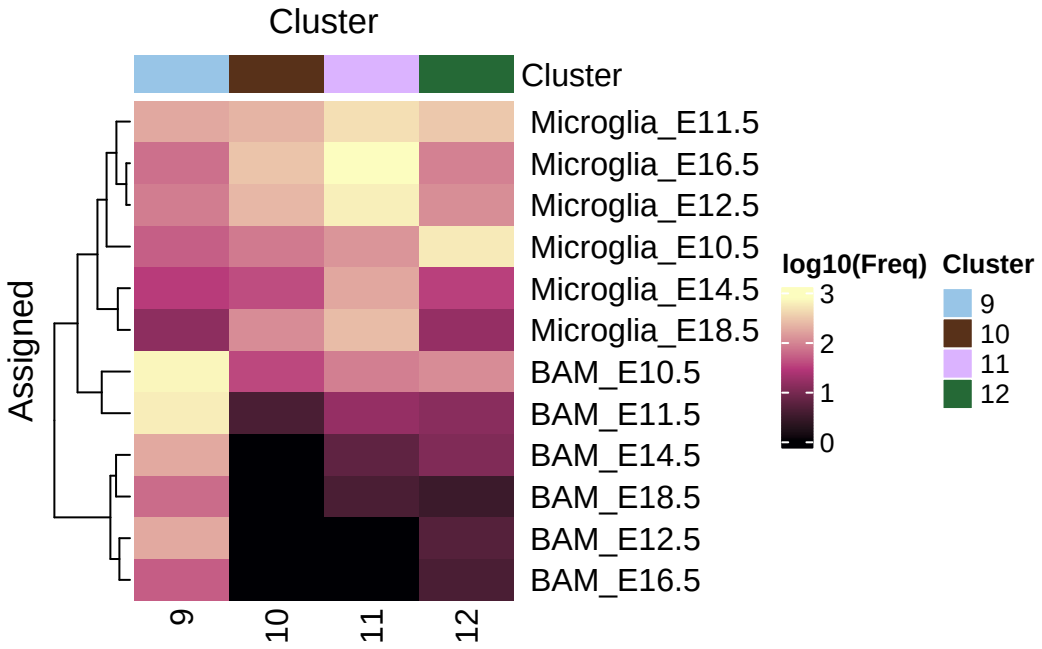
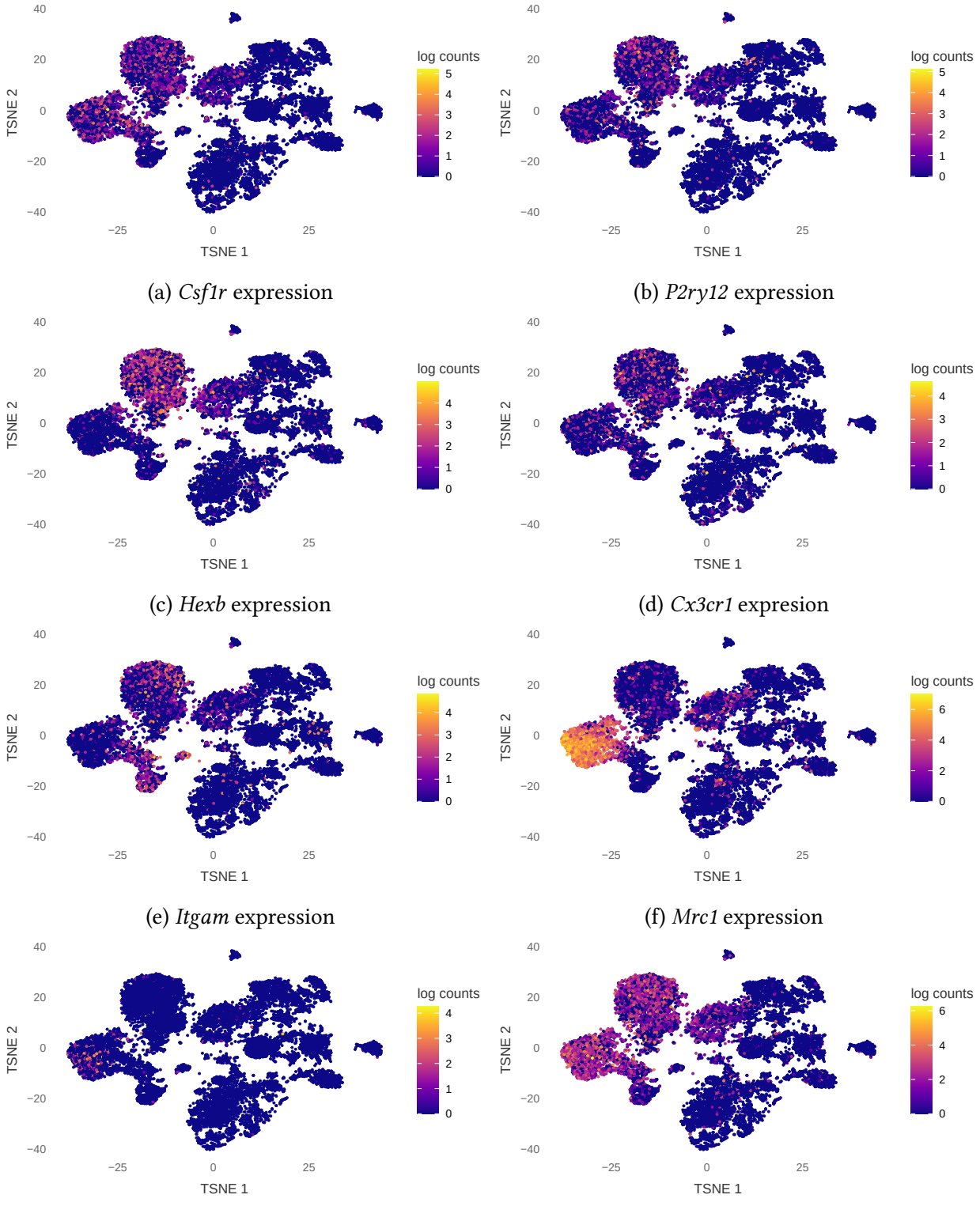


Figure 3.28: Heatmap showing the frequency of cells from a given myeloid cluster being assigned to a given reference cell type label from the Utz et al. 2020 data set.

To further explore and confirm the cell type identities of clusters, the expression of canonical microglia, BAM, monocyte, and ependymal markers was examined. High expression of *Mrc1* clearly delineated cluster 9 as BAM, while *P2ry12* and *Hexb* expression patterns suggest clusters 10 and 11 are microglia (Figure 3.29). These cell type labels are in line with the findings from the similarity analysis with the Utz et al. (2020) reference data. High expression of *Ccr2* and *Ptprc* and low expression of *Mertk* indicate cluster 12 may represent monocytes, as shown in [Figure 3.30](Laar et al., 2016). A subset of cells in cluster 12 also expressed MHC Class II proteins (e.g. *H2-Aa*). Cluster 1 was identified as epithelial ependymal cells, as demonstrated by expression of marker genes *Htr2c*, *Foxj1*, *Dynlrb2*, and *Cfap45* (Dani et al., 2021; MacDonald et al., 2021). The cell type identity labels and number of cells assigned to each cluster are reported in Figure 3.32 and Table 3.7.



(a) *Csf1r* expression

(b) *P2ry12* expression

(c) *Hexb* expression

(d) *Cx3cr1* expresion

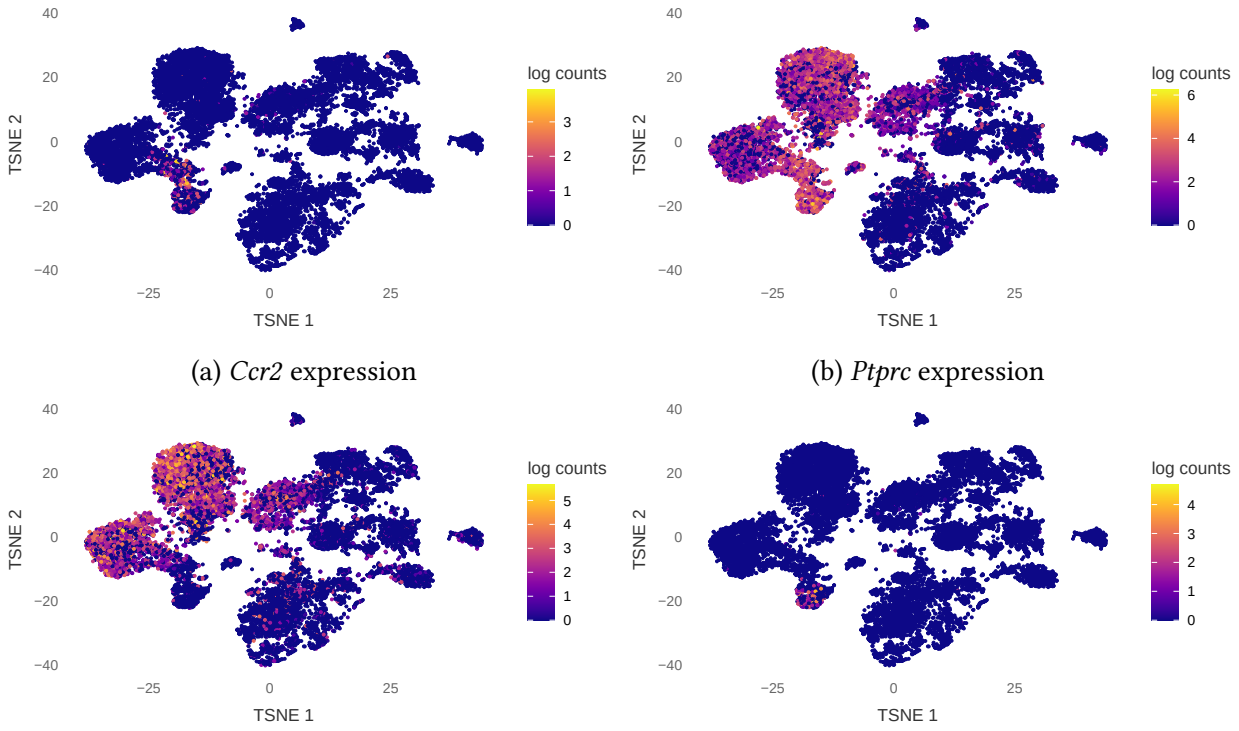
(e) *Itgam* expression

(f) *Mrc1* expression

(g) *Lyve1* expression

(h) *Adgre1* expression

Figure 3.29: Expression of canonical myeloid, microglial, and BAM marker genes differentiate myeloid clusters.



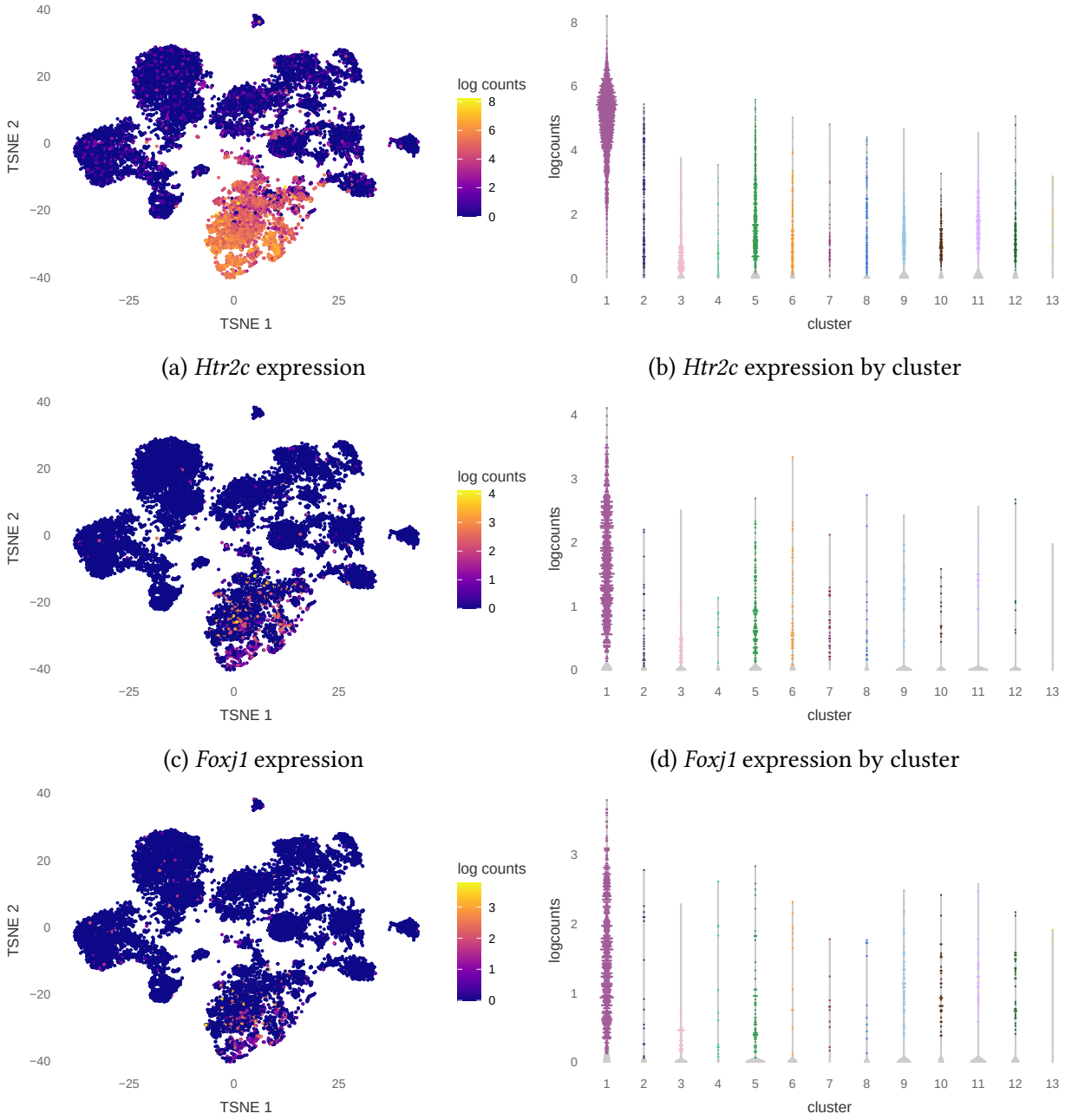
(a) *Ccr2* expression

(b) *Ptprc* expression

(c) *Mertk* expression

(d) *H2-Aa* expression

Figure 3.30: Expression of marker genes indicates monocyte identity for cells in cluster 10.



(a) *Htr2c* expression

(b) *Htr2c* expression by cluster

(c) *Foxj1* expression

(d) *Foxj1* expression by cluster

(e) *Cfap45* expression

(f) *Cfap45* expression by cluster

Figure 3.31: Expression of marker genes suggests ependymal cell identity for cells in cluster 1. Expression of three ependymal marker genes are visualized via t-SNE plot and via dot plot. The dot plots are separated by cluster, and each colored point represents a cell expressing the gene (non-zero counts) while the grey violin plot in the background for each cluster indicates the distribution of expression for all cells, including the proportion of cells not expressing the gene.

Table 3.7: Number of cells per cluster.

Cell Type	Number of Cells
BAM	2197
endothelial	373
ependymal	3947
microglia_1	1009
microglia_2	2552
monocytes	1270
neurons	2568
neutrophils	178
notochord	170
NPCs_neuroblasts	675
NPCs_neuroepithelial	1246
NPCs_radial glia	894
unassigned	1386

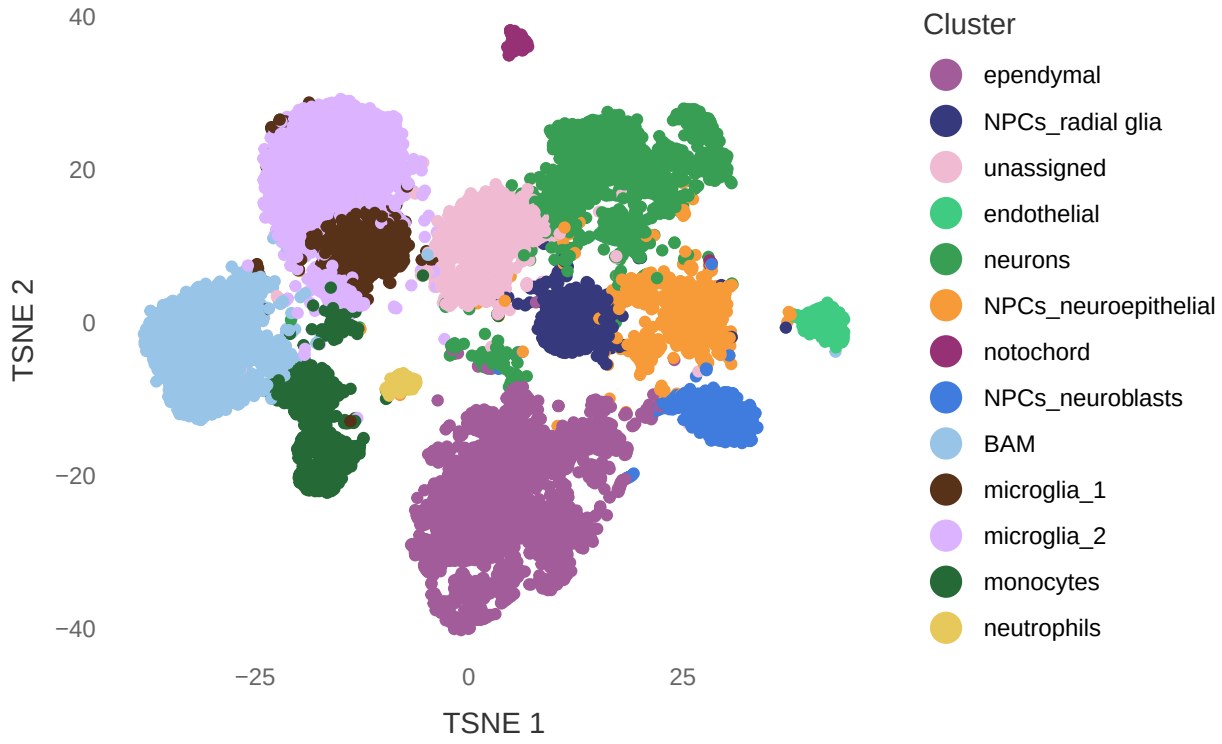
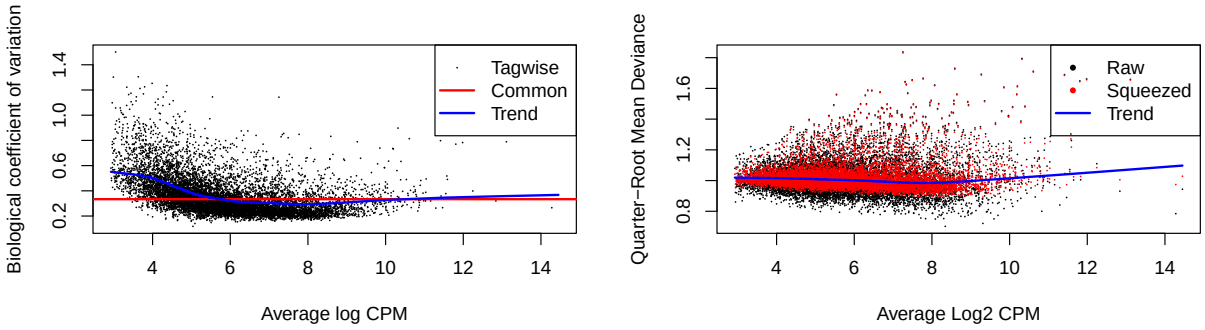


Figure 3.32: tSNE visualization of single-cell RNA expression showing clustering of cells. The cell type labels assigned to each cluster are shown, with cells colored by cell type.

3.3.7 Female Ependymal Cells and Male Neural Progenitor Cells Demonstrated Differential Gene Expression in Response to Poly(I:C)

Single cell counts for each sample were summed to create "pseudo bulk" samples within each cell type cluster we identified. This reduced sparsity and variability in the data, and allowed the application of methods developed for microarray and RNAseq to compare expression between experimental groups across replicates. Cells with unknown sex were excluded from pseudo bulk summation, and samples with fewer than 3 cells for a given cell type were excluded from analysis. The `edgeR` package was used to normalize the data, estimate dispersion, generate a fit, and test for differentially expressed genes (DEGs) (Figure 3.33). After filtering out sex-specific Y or X chromosome genes in comparisons of female and male groups (differential expression would be expected), 62 differentially expressed genes were found across cell types (Table 3.8). Surprisingly, over half of the DEGs were from ependymal cells (Figure 3.34).



(a) (b)

Figure 3.33: Plots of biological coefficient of variation and quasi-likelihood dispersion

Table 3.8: Differentially expressed genes between experimental groups in specific cell types. Genes with a Benjamini Hochberg adjusted p-value > 0.10 are reported.

gene_name	logFC	logCPM	F	PValue	FDR	Contrast	cell_type
Spsb1	5.119013	4.774473	21.58320	0.0000125	0.0808286	M_12_PvsV	BAM
Prex2	3.978034	4.315708	17.90845	0.0000565	0.0455894	V_12_MvsF	BAM
Thrb	-3.057704	5.978901	25.17237	0.0000080	0.0573228	F_12_PvsV	ependymal
Wnt5a	-2.601322	6.445075	23.08086	0.0000229	0.0573228	F_12_PvsV	ependymal
Synpo2	-6.297344	4.418278	21.59380	0.0000232	0.0573228	F_12_PvsV	ependymal
Col23a1	-1.815646	7.246333	21.66419	0.0000241	0.0573228	F_12_PvsV	ependymal
Zfp385b	-1.911252	6.927807	20.24189	0.0000327	0.0621696	F_12_PvsV	ependymal
Rian	-2.577768	7.241951	21.47634	0.0000426	0.0621844	F_12_PvsV	ependymal
Glul	-1.599455	6.835322	18.94234	0.0000484	0.0621844	F_12_PvsV	ependymal
Zranb3	-2.130496	5.803981	18.75094	0.0000524	0.0621844	F_12_PvsV	ependymal
Pde1a	4.334155	4.365359	31.36124	0.0000005	0.0043741	F_13_PvsV	ependymal
Slc9a9	3.545595	6.759191	29.27658	0.0000045	0.0215025	F_13_PvsV	ependymal
Fyb	3.135035	5.183789	23.48134	0.0000112	0.0355583	F_13_PvsV	ependymal
Adgre1	3.469268	4.923971	22.56534	0.0000161	0.0383602	F_13_PvsV	ependymal
Rasgrp3	3.099225	4.896082	21.15605	0.0000230	0.0421862	F_13_PvsV	ependymal
Cd44	2.773249	5.293825	21.12437	0.0000266	0.0421862	F_13_PvsV	ependymal
Vav3	2.472204	5.344550	19.17428	0.0000497	0.0590613	F_13_PvsV	ependymal
Inpp5d	3.225056	5.838804	20.96538	0.0000497	0.0590613	F_13_PvsV	ependymal
Mef2c	2.298823	6.758086	19.67091	0.0000658	0.0600084	F_13_PvsV	ependymal
Pik3ap1	2.844808	4.843754	18.37768	0.0000672	0.0600084	F_13_PvsV	ependymal
F13a1	3.234347	6.072618	20.35093	0.0000695	0.0600084	F_13_PvsV	ependymal
Col23a1	-2.192479	7.246333	27.77499	0.0000029	0.0093908	V_12_MvsF	ependymal
Cdh12	-2.726974	6.669877	22.92254	0.0000248	0.0393061	V_12_MvsF	ependymal
Man1a	-1.603324	7.128284	18.09982	0.0000743	0.0784454	V_12_MvsF	ependymal
Pde10a	-2.553924	6.090976	18.63668	0.0000964	0.0824436	V_12_MvsF	ependymal

(continued)

gene_name	logFC	logCPM	F	PValue	FDR	Contrast	cell_type
Gpr39	-5.968709	4.889754	18.37234	0.0001020	0.0824436	V_12_MvsF	ependymal
Pde6c	-2.522584	5.228702	17.24807	0.0001041	0.0824436	V_12_MvsF	ependymal
Ccbe1	-3.507854	6.046004	18.53004	0.0001303	0.0863225	V_12_MvsF	ependymal
Rhobtb1	-1.608763	6.191001	16.38375	0.0001395	0.0863225	V_12_MvsF	ependymal
Pla2g4a	-2.451101	5.125337	16.50489	0.0001456	0.0863225	V_12_MvsF	ependymal
1700011I03Rik	-5.642008	3.522968	16.37806	0.0001552	0.0863225	V_12_MvsF	ependymal
Gm36879	-2.619535	5.075620	16.50336	0.0001600	0.0863225	V_12_MvsF	ependymal
Sema3d	-3.937175	3.856273	16.00741	0.0001635	0.0863225	V_12_MvsF	ependymal
Tpd52l1	-1.510718	7.145631	16.26072	0.0001750	0.0874936	V_12_MvsF	ependymal
Adamtsl1	-1.540999	9.196171	16.16150	0.0002154	0.0983066	V_12_MvsF	ependymal
P3h2	-2.715690	5.468450	16.34262	0.0002227	0.0983066	V_12_MvsF	ependymal
Cobll1	-1.355328	7.148280	15.25533	0.0002276	0.0983066	V_12_MvsF	ependymal
Pde1a	-5.354701	4.365359	21.15173	0.0000200	0.0682673	Tx_13_MvsF	ependymal
Inpp5d	-4.698667	5.838804	23.04775	0.0000251	0.0682673	Tx_13_MvsF	ependymal
Fyb	-4.073524	5.183789	20.68306	0.0000315	0.0682673	Tx_13_MvsF	ependymal
Rasgrp3	-4.155696	4.896082	20.16081	0.0000338	0.0682673	Tx_13_MvsF	ependymal
Adgre1	-4.666674	4.923971	20.40181	0.0000359	0.0682673	Tx_13_MvsF	ependymal
Trerf1	2.154516	6.921657	28.12782	0.0000009	0.0043379	M_12_PvsV	microglia_1
Olfm4	9.262536	5.151437	22.85217	0.0000083	0.0393820	Tx_13_MvsF	microglia_1
Ttn	5.235994	5.659351	17.26579	0.0001151	0.0960553	V_12_MvsF	monocytes
Nos1ap	-2.263512	8.161648	22.20734	0.0000245	0.0671596	V_12_MvsF	NPCs_neuroblasts
Ank1	5.473886	5.391999	25.64784	0.0000023	0.0197162	F_12_PvsV	NPCs_neuroepithelial
Gm15564	9.080824	8.573289	26.66818	0.0000101	0.0735099	M_12_PvsV	NPCs_neuroepithelial
Dnah9	-2.858087	6.543662	20.46868	0.0000175	0.0735099	M_12_PvsV	NPCs_neuroepithelial
Ank1	-7.929961	5.391999	23.83728	0.0000048	0.0408079	Tx_12_MvsF	NPCs_neuroepithelial
Hmcn1	7.272722	6.000055	20.41274	0.0000156	0.0802752	M_12_PvsV	NPCs_radial glia
Dnah9	4.510688	5.897670	27.23362	0.0000008	0.0042047	M_13_PvsV	NPCs_radial glia

(continued)

gene_name	logFC	logCPM	F	PValue	FDR	Contrast	cell_type
Dnah11	3.564623	5.866246	20.47004	0.0000145	0.0373971	M_13_PvsV	NPCs_radial glia
Vat1l	2.054598	7.258596	18.24109	0.0000352	0.0538639	M_13_PvsV	NPCs_radial glia
Hydin	4.564338	5.664742	19.63346	0.0000418	0.0538639	M_13_PvsV	NPCs_radial glia
Gmnc	4.672060	5.900417	19.09128	0.0000792	0.0816539	M_13_PvsV	NPCs_radial glia
Gm7173	2.612915	6.330427	16.34668	0.0001047	0.0899666	M_13_PvsV	NPCs_radial glia
Gm20754	-4.603845	5.610314	26.44498	0.0000019	0.0097839	V_13_MvsF	NPCs_radial glia
Ncoa3	-2.288642	6.871665	19.63629	0.0000174	0.0897280	Tx	NPCs_radial glia
mt-Nd2	-2.547695	4.826605	23.15335	0.0000098	0.0999470	F_12_PvsV	unassigned
Xist	-6.636877	10.279349	27.06824	0.0000091	0.0931431	M_13_PvsV	unassigned
Cacnb2	-1.314854	8.367402	25.10040	0.0000047	0.0481744	V_12_MvsF	unassigned

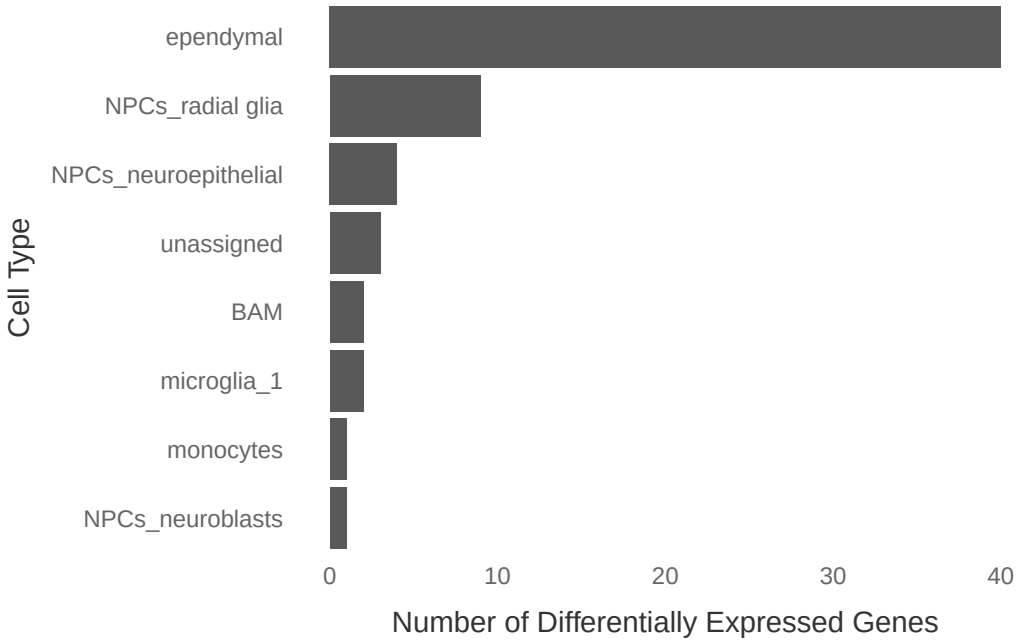


Figure 3.34: Number of differentially expressed genes (DEGs) by cell type. DEGs from all contrasts (comparisons between experimental groups) are included.

3.3.8 Gene Set Analysis Identified Immune Response in Ependymal Cells and Cilia Biology in Radial Glia as Key Pathways

To better understand the differential expression we observed in our data, we used gene set analysis to examine the molecular and cell pathways related to the sets of DEGs. We examined all DEG sets grouped by cell type and contrast (experimental comparison), and found significant results (with a p value < 0.05 after Benjamini Hochberg correction) for two sets: DEGs between Poly(I:C) and vehicle treated E13.5 females in ependymal cells, and DEGs between Poly(I:C) and vehicle treated E13.5 males from the "NPCs_radial glia" labelled cell cluster. 66 significant GO terms were found for the female ependymal cell gene set and 18 for the male NPC gene set (Table 3.9, Table 3.10). Only the top 25 significantly enriched terms are shown for female ependymal cells.

Table 3.9: Results of gene ontology enrichment analysis on differentially expressed genes in ependymal cells from treated compared to untreated E13.5 females. This table includes the top 25 most significantly enriched GO terms by Benjamini Hochberg adjusted p-value.

ID	Description	p-value	geneID
GO:0050776	regulation of immune response	0.0006	Fyb, Cd44, Vav3, Inpp5d, Mef2c, Pik3ap1
GO:0050670	regulation of lymphocyte proliferation	0.0011	Cd44, Vav3, Inpp5d, Mef2c
GO:0032944	regulation of mononuclear cell proliferation	0.0011	Cd44, Vav3, Inpp5d, Mef2c
GO:0070663	regulation of leukocyte proliferation	0.0011	Cd44, Vav3, Inpp5d, Mef2c
GO:0030888	regulation of B cell proliferation	0.0011	Vav3, Inpp5d, Mef2c
GO:0046651	lymphocyte proliferation	0.0014	Cd44, Vav3, Inpp5d, Mef2c
GO:0032943	mononuclear cell proliferation	0.0014	Cd44, Vav3, Inpp5d, Mef2c
GO:0042100	B cell proliferation	0.0014	Vav3, Inpp5d, Mef2c
GO:0050871	positive regulation of B cell activation	0.0014	Vav3, Inpp5d, Mef2c
GO:0070661	leukocyte proliferation	0.0014	Cd44, Vav3, Inpp5d, Mef2c
GO:0002250	adaptive immune response	0.0014	Adgre1, Cd44, Inpp5d, Mef2c
GO:0002764	immune response-regulating signaling pathway	0.0020	Fyb, Vav3, Mef2c, Pik3ap1
GO:0002684	positive regulation of immune system process	0.0027	Fyb, Cd44, Vav3, Inpp5d, Mef2c
GO:0050778	positive regulation of immune response	0.0033	Fyb, Cd44, Vav3, Mef2c
GO:0050864	regulation of B cell activation	0.0038	Vav3, Inpp5d, Mef2c
GO:0050851	antigen receptor-mediated signaling pathway	0.0045	Fyb, Vav3, Mef2c
GO:0051249	regulation of lymphocyte activation	0.0050	Cd44, Vav3, Inpp5d, Mef2c
GO:0006925	inflammatory cell apoptotic process	0.0053	Cd44, Mef2c
GO:0002429	immune response-activating cell surface receptor signaling pathway	0.0053	Fyb, Vav3, Mef2c
GO:0002757	immune response-activating signal transduction	0.0053	Fyb, Vav3, Mef2c
GO:0002768	immune response-regulating cell surface receptor signaling pathway	0.0053	Fyb, Vav3, Mef2c
GO:0030224	monocyte differentiation	0.0065	Inpp5d, Mef2c
GO:2000108	positive regulation of leukocyte apoptotic process	0.0068	Cd44, Mef2c
GO:0002694	regulation of leukocyte activation	0.0068	Cd44, Vav3, Inpp5d, Mef2c
GO:0030890	positive regulation of B cell proliferation	0.0079	Vav3, Mef2c

Table 3.10: Results of gene ontology enrichment analysis on differentially expressed genes in the NPCs/radial glial cell cluster from Poly(I:C) treated compared to untreated E13.5 males. This table includes GO terms with enrichment p-values < 0.05 after Benjamini Hochberg correction.

ID	Description	p-value	geneID
GO:0003341	cilium movement	0.0000	Dnah9, Dnah11, Hydin, Cfap47
GO:0007018	microtubule-based movement	0.0001	Dnah9, Dnah11, Hydin, Cfap47
GO:0003351	epithelial cilium movement involved in extracellular fluid movement	0.0014	Dnah9, Dnah11
GO:0006858	extracellular transport	0.0014	Dnah9, Dnah11
GO:0007017	microtubule-based process	0.0014	Dnah9, Dnah11, Hydin, Cfap47
GO:0060271	cilium assembly	0.0014	Hydin, Gmnc, Cfap47
GO:0030317	flagellated sperm motility	0.0014	Dnah11, Cfap47
GO:0097722	sperm motility	0.0014	Dnah11, Cfap47
GO:0035082	axoneme assembly	0.0014	Hydin, Cfap47
GO:0060294	cilium movement involved in cell motility	0.0014	Dnah11, Cfap47
GO:0044782	cilium organization	0.0014	Hydin, Gmnc, Cfap47
GO:0001539	cilium or flagellum-dependent cell motility	0.0014	Dnah11, Cfap47
GO:0060285	cilium-dependent cell motility	0.0014	Dnah11, Cfap47
GO:0001578	microtubule bundle formation	0.0032	Hydin, Cfap47
GO:0120031	plasma membrane bounded cell projection assembly	0.0045	Hydin, Gmnc, Cfap47
GO:0030031	cell projection assembly	0.0045	Hydin, Gmnc, Cfap47
GO:0070925	organelle assembly	0.0144	Hydin, Gmnc, Cfap47
GO:0099111	microtubule-based transport	0.0144	Dnah9, Dnah11

3.4 Discussion

Few studies have examined fetal brain cell responses to MIA at single cell resolution and accounting for sex differences. Prior work from Kalish et al. (2021) examined single cell transcriptomes in embryonic brain at E14.5 and E18.5 after E12.5 Poly(I:C) administration. They found many differentially expressed genes in E14.5 males across multiple neuronal cell types, primarily related to metabolism and protein translation. This group showed that the integrated stress response was activated in male MIA offspring downstream of maternal IL-17a, and blockade of this response could rescue social preference and marble burying behavioral phenotypes in their model. However, the mechanism by which maternal IL-17a and/or other MIA-induced immune mediators lead to a stress response in male neural cells remains unclear. In order to investigate the acute response to MIA in fetal brain cells and capture early molecular events, we examined the transcriptome of brain cells 6 and 24hr after MIA.

3.4.1 Response to Maternal Immune Activation in Fetal Brain Myeloid Cells

We induced MIA using a 10mg/kg dose of Poly(I:C), and observed sickness phenotypes in injected dams. Surprisingly, despite the apparent systemic effect on the maternal immune system, we did not see the expected strong transcriptional response in fetal brain myeloid cells. When comparing gene expression between groups that did or did not receive Poly(I:C) stimulation, we observed few DEGs in myeloid cells: 1 in BAM, and 2 in the microglia_1 cluster. In BAM, E12.5 males treated with Poly(I:C) had 5-fold upregulation of *Spsb1* transcripts compared to untreated. Spsb1 is a substrate adaptor component of a ubiquitin ligase complex involved in protein degradation (Nishiya et al., 2011). This adaptor has been shown to specifically negatively regulate NF-κB activation, so its upregulation may indicate negative regulation or resolution of the immune response in male BAM at E12.5 (Georgana and Maluquer de Motes, 2020). In microglia, treated E12.5 males had 2-fold upregulation of the transcriptional regulator *Trerf1* compared to untreated. Trerf1 activates transcription of Cyp11a1, an enzyme involved in steroid hormone synethesis that converts cholesterol to pegnenolone (Gizard et al., 2001). This finding represents an interesting link between response to Poly(I:C) and increased sex steroid metabolism in male microglia that warrants further investigation. In comparing response to treatment by sex in the microglia_1 cluster, *Olfm4* had 9-fold greater induction in response to treatment in males compared to females at E13.5. Like Spsb1, Olfm4 is a negative regulator of NF-κB activation, and expression of Olfm4 is regulated by granulocyte colony-stimulating factor and NF-κB, which binds at the *Olfm4* promoter (Chin et al., 2008; Gong et al., 2021). Increased *Olfm4* expression after Poly(I:C) in E13.5 males may indicate prior immune activation of the microglia and activation of NF-κB, which potentially led to upregulation of *Olfm4*.

We expected to see differentially expressed genes related to the immune response in myeloid cells given our prior work, which used a lower 2mg/kg dose of Poly(I:C) and a similar injection and collection schedule (E12.5 injection, E13.5 sample collection after 24hr). Using this paradigm our

lab previously observed upregulation of immune response pathways in a BAM-like cell cluster after MIA (Nichols et al., 2020). Matcovitch-Natan et al. (2016) also observed transcriptional shifts in fetal microglia after mid-gestation Poly(I:C) MIA. Here, we observed only a few differentially expressed genes in BAM and microglia in response to Poly(I:C), two of which are related to the immune response as NF-κB response and regulator genes. The discrepancy between studies may be due to differences in replicate number, sample collection and library preparation methods, sequencing depth, or variability in MIA induction due to animal microbiome or Poly(I:C) reagent batch differences.

3.4.2 Response to MIA in Ependymal and Neural Progenitor Cell Populations

Interestingly, we observed larger numbers of differentially expressed genes between Poly(I:C) and vehicle treated groups in three non-myeloid cell types: ependymal cells, and two of the neural progenitor cell clusters, labeled "NPCs_radial glia" and "NPCs_neuroepithelial". Through gene set analysis we found significantly enriched gene ontology terms related to immune response and immune cell proliferation in the E13.5 female ependymal cells, and terms related to cell transport, motility, and cilia in the E13.5 male radial glia cluster.

To our knowledge, this is the first report of fetal ependymal cells responding to Poly(I:C) maternal immune activation, and additionally the first reported observation of a sex difference in fetal ependymal cells. Ependymal cells are epithelial cells which differentiate from radial glia in the brain and form a layer along the surface of the ventricles. Ependymal cells have cilia facing the ventricular space that beat to direct flow of cerebrospinal fluid, and may also be involved in trophic support of cells in the developing neuroepithelium (Del Bigio, 2009). Though ependymal cells have important roles in neurodevelopment, their association with neuropsychiatric conditions in adult brain is less clear and largely unstudied (Comte et al., 2012). Ependymal cells are known to respond to immune stimuli, and human fetal ependymal disruption has been reported after maternal cytomegalovirus and Zika virus infection (Mishra and Teale, 2012; Adams Waldorf et al., 2018; Lanna et al., 2021). Immune alterations in ependymal cells after MIA could impact the flow or chemical composition of CSF, ventricular pressure, or local signals in the neuroepithelium and subventricular zone, thereby impacting neurogenesis (Lehtinen and Walsh, 2011; Jiménez et al., 2014). Intriguingly, upregulation of immune response genes in ependymal cells after Poly(I:C) was only observed in females. This difference may represent underlying differences in the immune responsiveness of female and male ependymal cells, or could be due to upstream fetal sex-specific immune signals. We did observe 16 differentially expressed genes between female and male vehicle-treated ependymal cells at E12.5. This suggests that baseline sex differences exist at this time point. No significant GO terms were found for this gene set, which may indicate sex-differential genes have diverse functions in embryonic ependymal cells.

Gene set analysis of Poly(I:C) responsive differentially expressed genes in E13.5 male samples from the "NPCs_radial glia" cluster identified cell transport and cilium processes as key pathways. Radial glia are progenitor cells for neurons and ependymal cells, and divide symmetrically

and asymmetrically to proliferate and produce neurons. Radial glia span the neuroepithelium from the ventricle on the apical side to the pia surface on the basal side, and form the scaffold for migrating neuroblasts. In addition to these key roles in generation and localization of neurons, radial glia regulate neural connectivity (Casingal et al., 2022). The primary cilia of radial glia are important in regulating cell polarity and therefore proliferation and the ultimate size and structure of the cortex (Wilsch-Bräuninger and Huttner, 2021). Upregulation of these genes in males after Poly(I:C) could represent disruption of proliferation in radial glia. In a model of LPS-mediated MIA, proliferation of radial glia was decreased in males 2hr after administration of LPS at E12.5, but was not significantly different in females (Braun et al., 2019). An alternative possibility is that cell cycle differences between samples led to the upregulation of these genes in treated compared to untreated groups, without relevance for the fetal brain response to Poly(I:C).

3.4.3 Caveats and Conclusions

The overall low number of differentially expressed genes we observed could be due to a number of factors. The number of replicates may not have been sufficient to capture a majority of DEGs, especially those with lower fold-change values (Schurch et al., 2016). Some replicates were removed during filtering steps due to low cell number or lack of representation from all experimental groups, leading to lower replicate numbers particularly for clusters with fewer cells. These factors could explain why more DEGs were found for the ependymal cell cluster, which is the cluster with the most cells (3947). However, the radial glia cluster (894 cells) still showed differential expression between specific groups, and when combining myeloid cell clusters into one set of pseudobulk samples, differentially expressed genes were still not observed. This specific MIA paradigm may also have a less pronounced or more cell-type specific immune response in the fetal brain compared to other protocols.

Our findings highlight new potential responder cell types of interest that may be important in the etiology of MIA phenotypes. Ependymal and radial glia cells showed sex-specific gene expression responses to MIA in our model. Validation of these pathways and responder cell types could contribute to our knowledge of neurodevelopmental disorders with environmental origins and/or sex differences.

Part II

Building Social Justice in STEM

4 Effective and Equitable Pedagogy from a Distance: Meeting the Challenge of Undergraduate Learning in Molecular and Cell Biology During the COVID-19 Pandemic

4.1 Introduction

In Spring 2020, the COVID-19 pandemic led to a shift from in-person to remote teaching and learning. As the U.S. Department of Education's Office for Civil Rights has reported, the pandemic exacerbated many inequalities already present in higher education, including barriers to access and achievement for low-income students, students of color, and students with disabilities (Office for Civil Rights, 2021). In order to aid my academic department (Molecular and Cell Biology at UC Berkeley, "MCB") in adapting to remote learning in an evidence-based and equitable way, I founded the MCB Distance Learning Task Force ("Task Force"). In the context of inequities in higher education compounded by the pandemic, our goal was to offer the highest quality and most equitable remote education to our students as a way to begin to address those inequities.

Necessary changes to education during the pandemic also provided an opportunity to implement new pedagogical methods as instructors and students adjusted to a new educational paradigm. During this window where instructors were seeking guidance, the Task Force sought to instill principles of well-established pedagogical improvements, applicable to both online and in-person learning, that are known to lead to improved learning outcomes and reduce opportunity gaps. We hope that some of the ideas we proposed and distributed will have continued relevance and implementation beyond remote learning and the pandemic.

4.2 Methods

I solicited members for the Task Force from multiple MCB stakeholder groups, resulting in 18 members (4 undergraduate students, 7 graduate students, 1 postdoc, 3 faculty, and 3 staff). Through a collaborative discussion process the Task Force collectively decided on three main

topics to focus on and formed three working groups: Student Experience Survey, Instructor Resources, and Training for Graduate Student Instructors. Over the course of 1-4 meetings per group, the working groups clarified their goals, delegated responsibilities and reviewed output.

4.2.1 Student Experience Survey Design

The Student Experience Survey working group designed research questions and disseminated a survey to MCB undergraduate students. The group was composed of 7 members, including MCB undergraduates and faculty, to incorporate a range of perspectives. We collectively designed 22 multiple choice, 7 free-response, and 9 demographic survey questions based on the problems and opportunities of remote learning identified by the larger Task Force. We collected data on accessibility, course organization, educational disparities, assessments/grading, and engagement and community. We included 7 short answer questions to gauge student concerns, experiences, and ideas for improvement. Demographic questions were designed based on the recommendations of Hughes et al. (2016).

4.2.2 Student Experience Survey Dissemination and Data Analysis

The survey was generated and disseminated using Qualtrics. The target population was MCB students who both took online MCB courses in Spring 2020, and planned to take online MCB courses in Fall 2020. Surveys were distributed by MCB faculty to students in both upper division and lower division MCB courses. Of the 336 students who responded, 134 fit our target population criteria. All these responses were analyzed, including from respondents who didn't complete the survey, though most students (122 out of 134) completed the survey from start to finish. Proportions for a given response were calculated using the total number of people who answered the associated question to account for partially completed surveys. Figures of quantitative data were made using the online Qualtrics software. Qualitative data were analyzed by two independent working group members and grouped into five main topics, and either paraphrased or quoted directly.

4.2.3 Literature Review and Creation of a Website of Recommendations for Instructors

The Instructor Resources working group, composed of 8 members, worked in pairs on specific research topics of interest determined by the group. Members did literature reviews of education and STEM (science, technology, engineering, and math) education research. The whole working group reviewed and edited findings before dissemination in the form of a "Teaching Remotely" website.

4.3 Results

4.3.1 Student Experience Survey

MCB undergraduate students were surveyed to investigate the impact of online learning in Spring 2020, to better understand student barriers and learning needs, and to gather student opinions on how the MCB department could improve instruction for Fall 2020. 134 student responses were analyzed after filtering for students meeting our criteria. The following sections summarize results of the survey and outline the recommendations we made to instructors and the MCB department leadership based on these results. Results are grouped into 5 topic categories, each of which include both quantitative data and paraphrased summaries of student free response answers. These categories are: 1) Impact of remote instruction on learning, 2) Improving the remote lecture experience, 3) Facilitating student interaction, 4) Accessibility, external difficulties, and student concerns, and 5) Exams and grades.

4.3.1.1 Impact of Remote Instruction on Learning

Slightly over half the students surveyed felt they did worse in their classes in Spring 2020 with remote learning compared to in person instruction (Figure 4.1). Around 20% of students reported doing better with remote learning compared to in person instruction while the remaining students felt they did equally well with both modes of learning. A number of student concerns were raised in relation to their performance, paraphrased as follows: 1) It's hard to maintain motivation, and concentrating on online classes for many hours a day is difficult, 2) It's hard to establish study groups, maintain social connection, and interact with professors remotely, 3) I'm concerned that I'm learning less online, particularly missing laboratory experience, 4) I'm concerned that instructors will make exams much harder to prevent cheating, but students will be under-prepared.

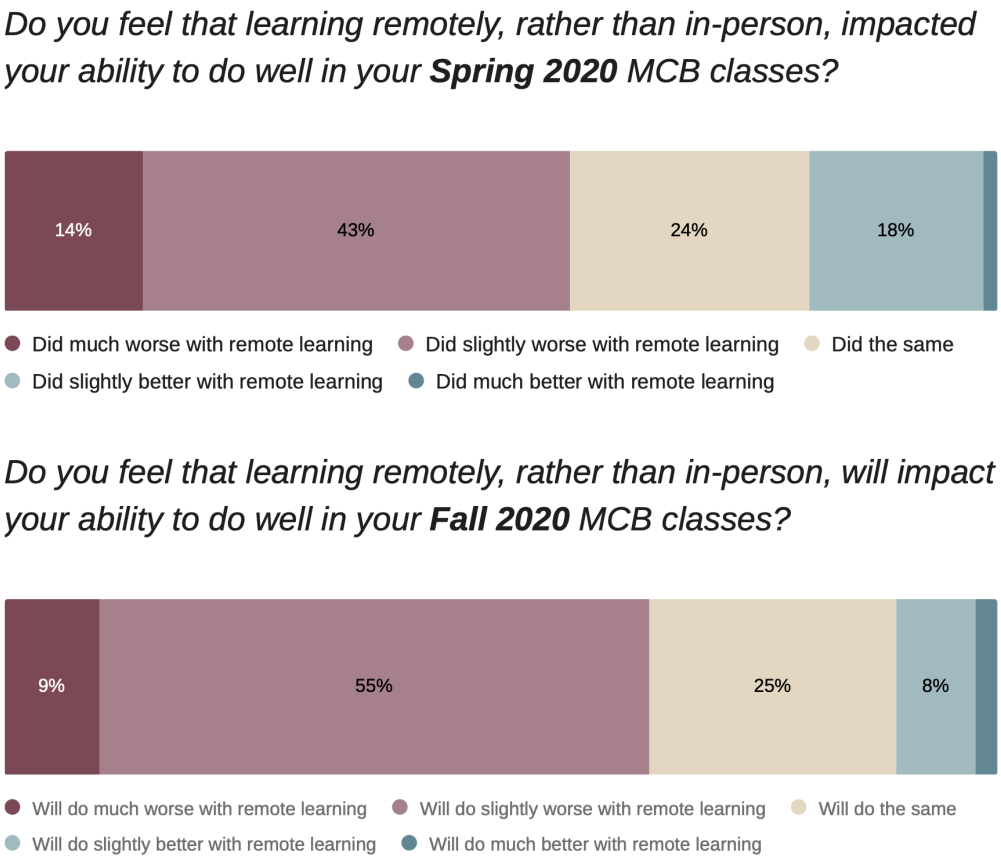


Figure 4.1: Student perception of their ability in Spring 2020 remote classes and expectation of ability in Fall 2020 remote classes.

4.3.1.2 Improving the Remote Lecture Experience and Student Interaction

The top two most engaging formats were office hours and live synchronous lecture (Figure 4.2, Figure 4.5). Students overwhelmingly preferred live or recorded video over audio-only lecture (Figure 4.2). Although 65% of students found live lecture engaging or very engaging, only 22% of students felt they will be able to attend synchronous classes consistently without any difficulties (Figure 4.3).

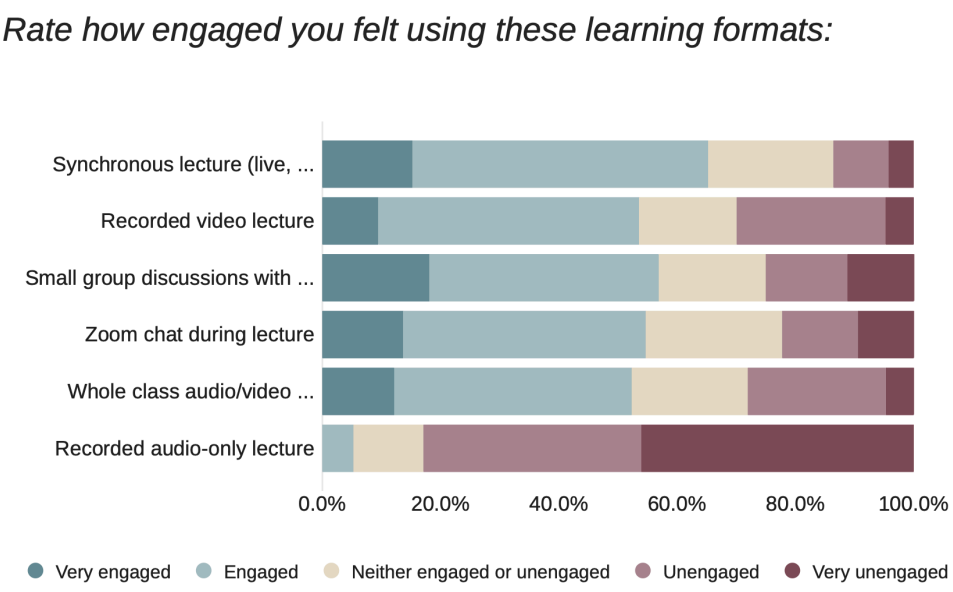


Figure 4.2: Student ratings of their engagement with different instructional formats.

Many students expected attending synchronous classes in Fall 2020 to be difficult for a variety of reasons (Figure 4.3). The majority of students expected distractions or noise in the home environment (60%), and students also cited family/home duties like childcare (30%), work shifts overlapping with class (29%), technology issues (28%), and time zone differences (19%) as likely issues. Some students expected to experience several of these issues at once.

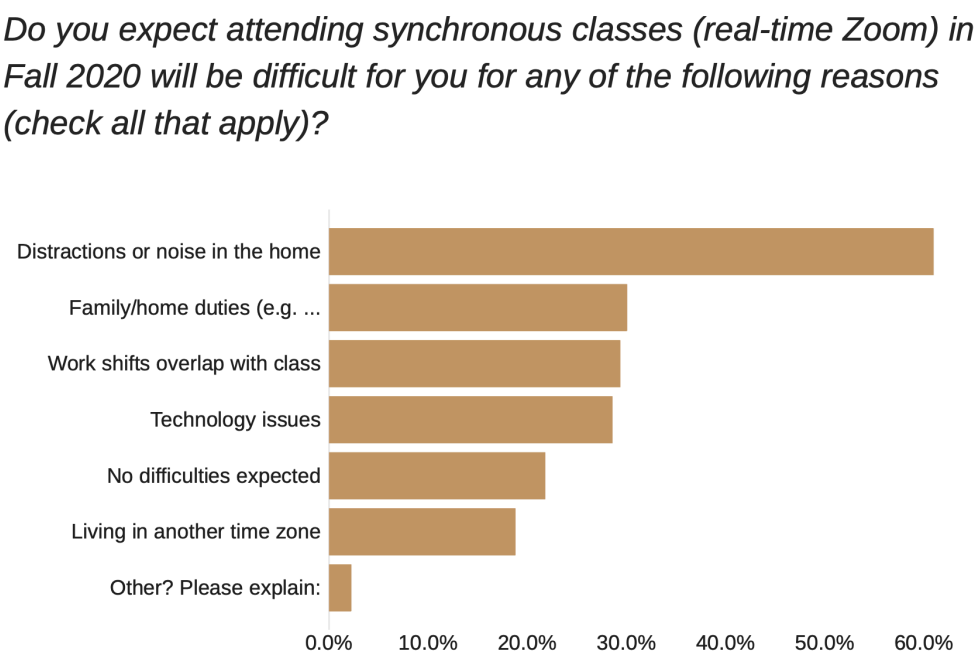


Figure 4.3: Expected reasons for difficulty attending synchronous classes in Fall 2020.

Students varied in how engaging they found interactions that occur during synchronous classes. Small group discussions with peers, Zoom chat during lecture, and whole class audio/video discussion were rated as engaging or very engaging for around half the students, with the caveat that around a quarter found these formats unengaging or very unengaging (Figure 4.2). These data and student comments suggest these formats need to be well-structured with clear instructions to be engaging for everyone.

Students offered comments and suggestions on ways to improve the remote learning environment, paraphrased here: 1) Offer both synchronous lecture and recordings, without making synchronous lecture mandatory unless interaction is an essential component, 2) Check WiFi connections and microphones for quality, 3) Having a second instructor monitor and answer questions in a live chat during lecture is very helpful, 4) Breakout rooms can be effective if clear instructions are provided, and 5) It's hard to motivate to watch Zoom recordings of lecture, so offering a participation quiz after each lecture could help students stay on track

4.3.1.3 Facilitating Student Interaction

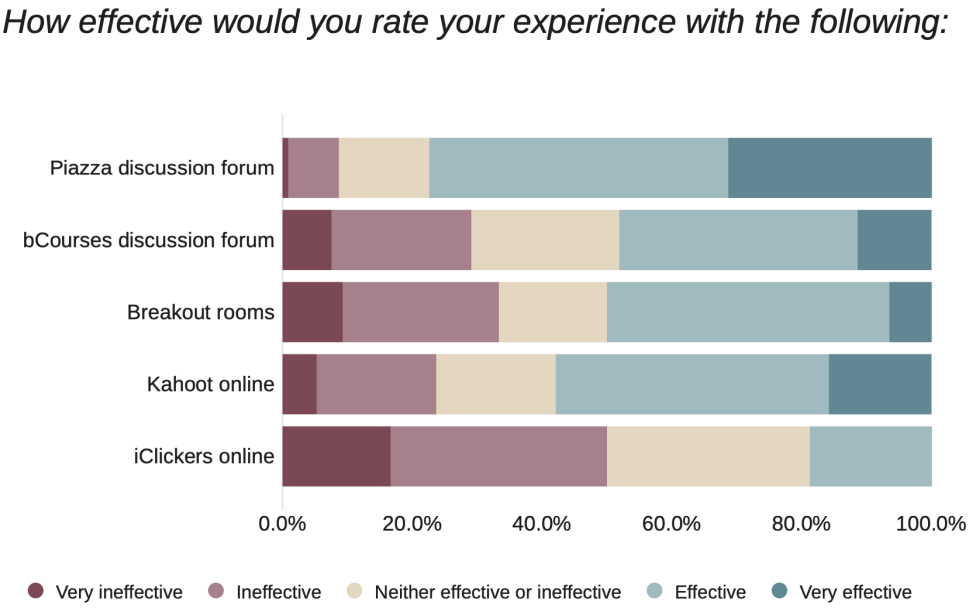


Figure 4.4: Students ranked the effectiveness of different technologies for promoting student interaction and discussion

Students found Piazza discussion forums by far the most effective technology for student interaction, with 32% of students rating it very effective and 46% rating it effective (Figure 4.4). Piazza was preferred compared to bCourses Discussion forum. Response to Zoom Breakout rooms was mixed, with ~50% rating them effective. Student comments suggested Breakout rooms with clear structure and instructions worked best. Kahoot was preferred over iClickers for live quiz polling, though only 30-40% of students had used these polling technologies.

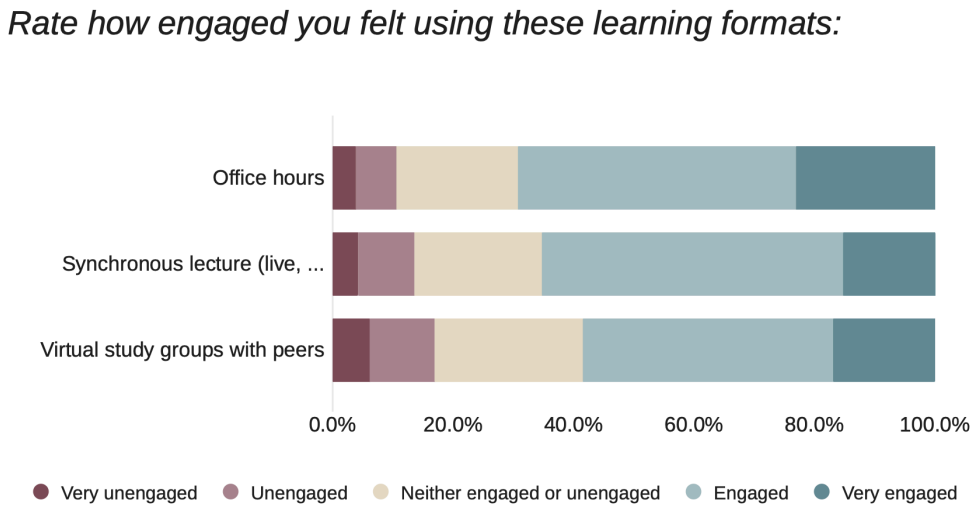


Figure 4.5: Students rated how engaged they felt during activities that may promote student interaction.

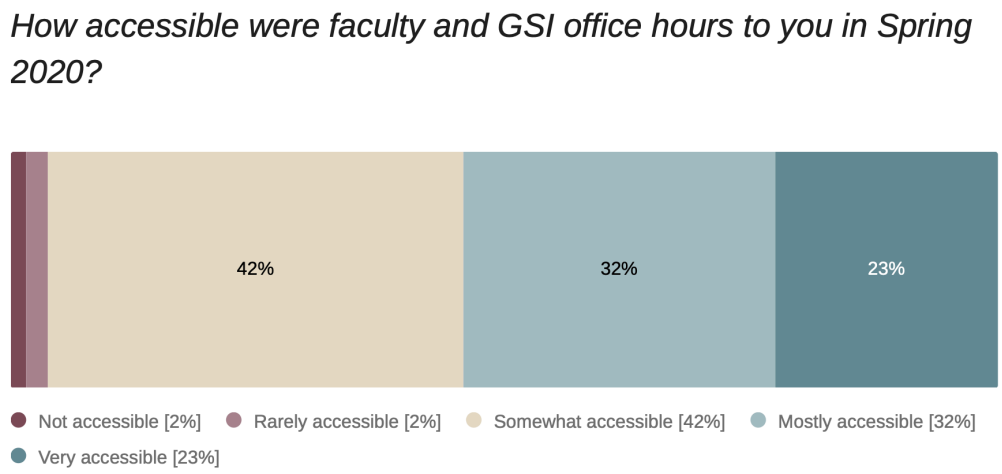


Figure 4.6: Students rated the accessbility of faculty and GSI office hours during Spring 2020.

Office hours were rated as engaging for most students (~70% engaging or very engaging), as were virtual study groups (~60%) (Figure 4.5). However many students did not participate in office hours or virtual study groups (18% and 49% marked not applicable, respectively). Office hours were mostly or very accessible to half of the students, somewhat accessible to 42%, and rarely or not accessible to 4% of students (Figure 4.6).

Student comments and suggestions related to student interaction are summarized and paraphrased as follows: 1) An instructor-monitored Piazza is extremely useful, 2) I really appreciate opportunities for extra interaction time with instructors and other students outside of class 3) Offer office hours and make sure they are available to students in different time zones

4.3.1.4 Improving Accessibility and Addressing Student Needs

Most students we surveyed have access to the technology they need for remote learning. A small percent of the students we surveyed have only very limited or somewhat reliable access to a computer (8%) or mobile phone (5%) (Figure 4.7). Internet connectivity is a broader issue, since most students reported some level of reduced access. A majority of students had either very limited access (1%), somewhat reliable (21%), or mostly reliable access (53%) to stable internet (Figure 4.8).

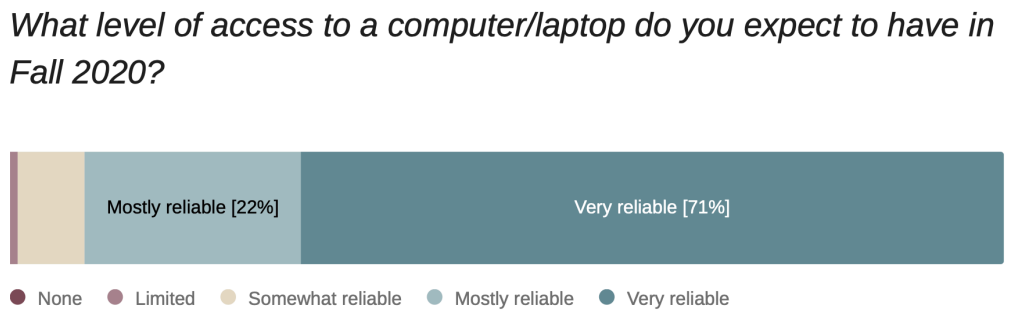


Figure 4.7: Students reported their expected level of access to a computer in Fall 2020.

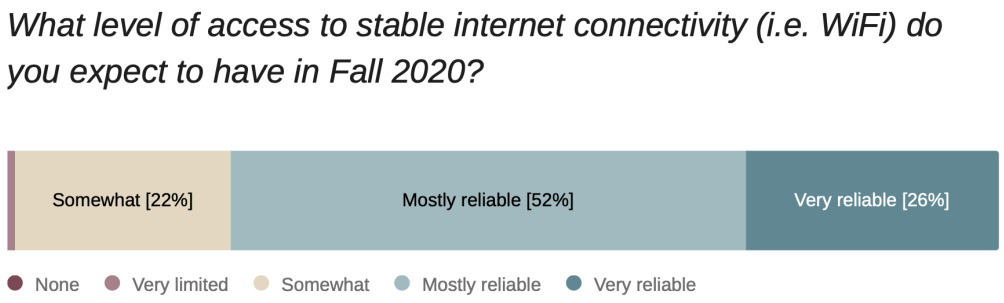


Figure 4.8: Students reported their expected level of access to the internet in Fall 2020.

While most students felt that instructions about course material, online assignments, and exams were relatively clear, there were still ~25% of students who felt confused about assignment details

and deadlines as well as navigating online exams (Figure 4.9). Fewer students (13-16%) found it confusing to locate course files, submit exams, and submit assignments.

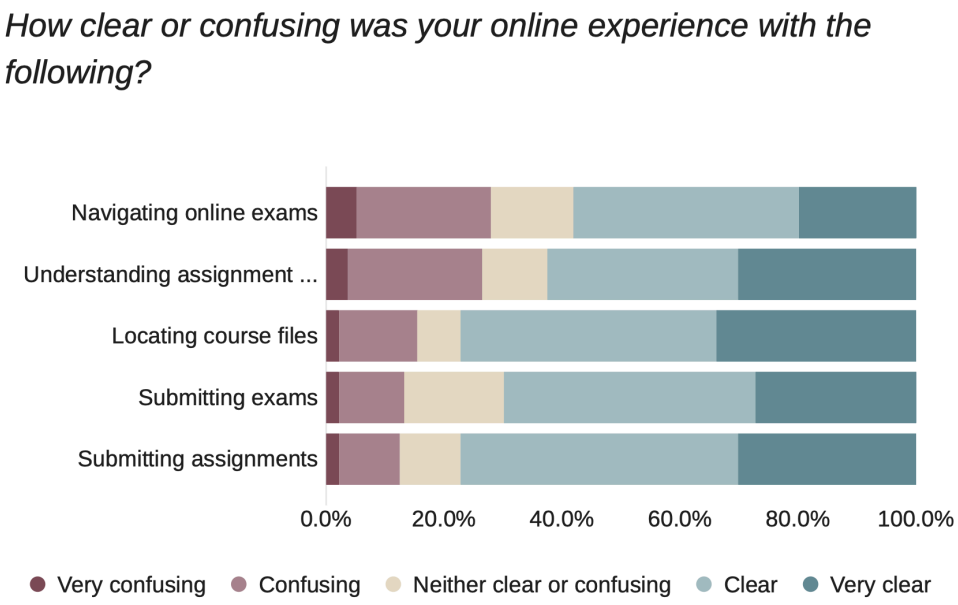


Figure 4.9: Students ranked how confusing they found different aspects of online learning.

One third of students answered that they will need financial assistance (33%), while a quarter need mental health services (24%) to participate in online courses (Figure 4.10). Additional services needed for students to participate are health services (16%), housing assistance (8%) and disability services (6%). Most students answered that they would need tutoring of some kind to succeed in their online courses (78%), though this number may be somewhat inflated because there was no "not applicable" or "none" option for this question.

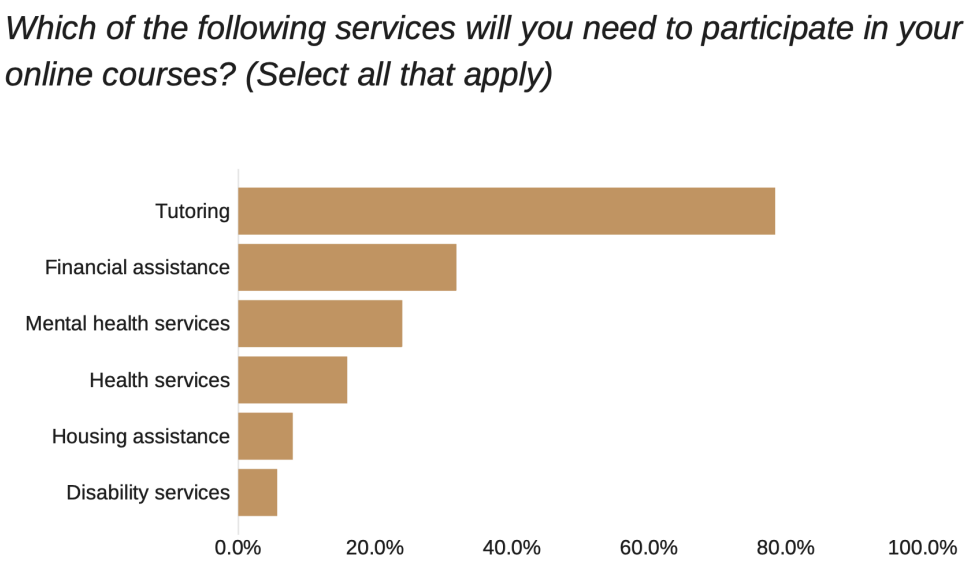


Figure 4.10: Students marked which services they would need to participate in online courses.

Over 70% of students reported that they would be working while taking classes, with 11% working 17 hours or more a week (Figure 4.11).

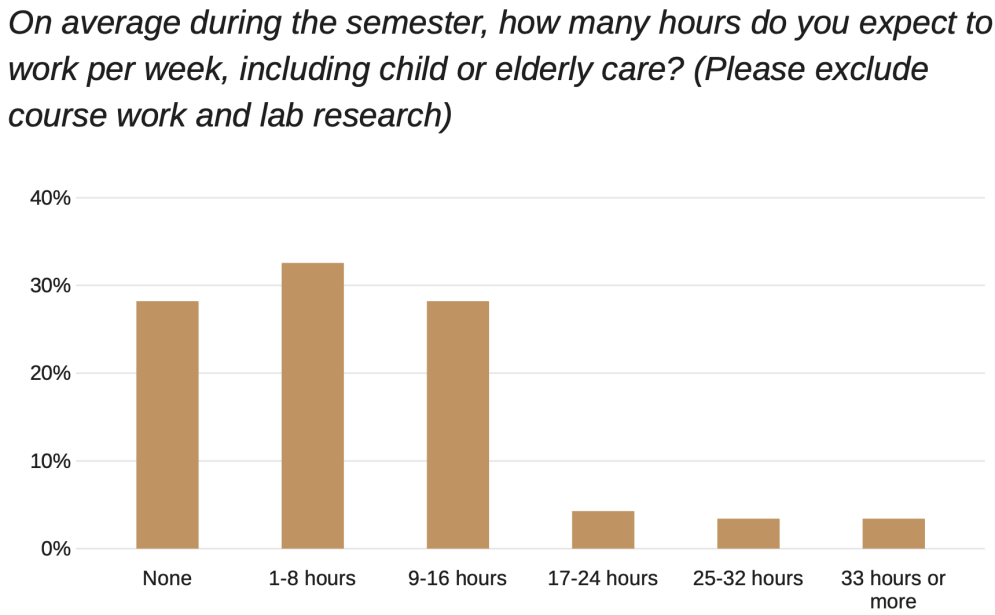


Figure 4.11: Hours per week students expect to work during the semester.

Students are interested in getting training in skills that will help them succeed with remote learning (Figure 4.12). 30% of students were interested in trainings on using learning technologies like Zoom, Gradescope, and Piazza (maybe, likely, or very likely to attend). Even more students were interested in trainings on remote learning study skills (64%) and building an inclusive online classroom community (63%), with significant proportions being very likely to attend (17% and 12%, respectively).

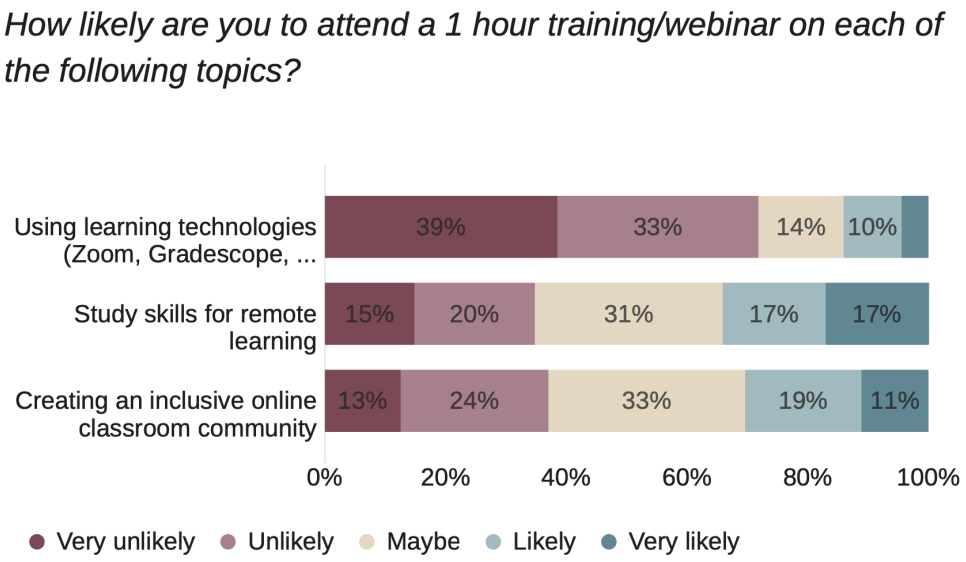


Figure 4.12: Student interest in attending training on technological, study, or community building skills

Students provided the following paraphrased comments and suggestions: 1) I appreciate when instructors provide study resources like practice exams so we can understand the change in question style and format, 2) Having recorded lectures is more equitable across time zones and easier to access if internet or computer use is unreliable, 3) It was very helpful when instructors showed support for students by giving reminders about mental health and self care, 4) Be understanding and flexible about difficult circumstances beyond the classroom, and grant extensions especially for health reasons, 5) Offer additional office hours or small group tutoring.

4.3.1.5 Exams and Grades

Three quarters of students felt that MCB major grading policies were communicated clearly, however some confusion still remained (Figure 4.13). Considering individual MCB classes, over half of the students felt that grading policies were communicated clearly, but almost 30% of students still felt that they were unclear (Figure 4.13).

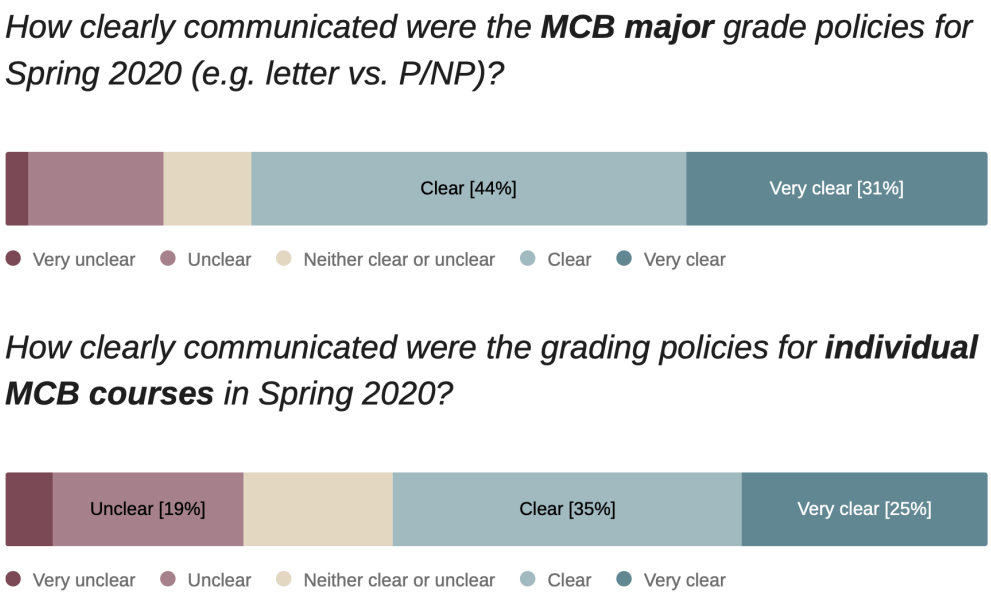


Figure 4.13: Students rated the clarity of communication about grade policies for the MCB major and individual MCB courses in Spring 2020.

Students had varying views on the importance of a Pass/No Pass grading option for MCB classes, from very important to not important at all (Figure 4.14). However, for the Letter Grade option, over 80% of students said that it was moderately or very important to them to have a letter grade option available. We recommend retaining the Pass/No Pass option since ~50% of students rated this option as moderately or very important for them.

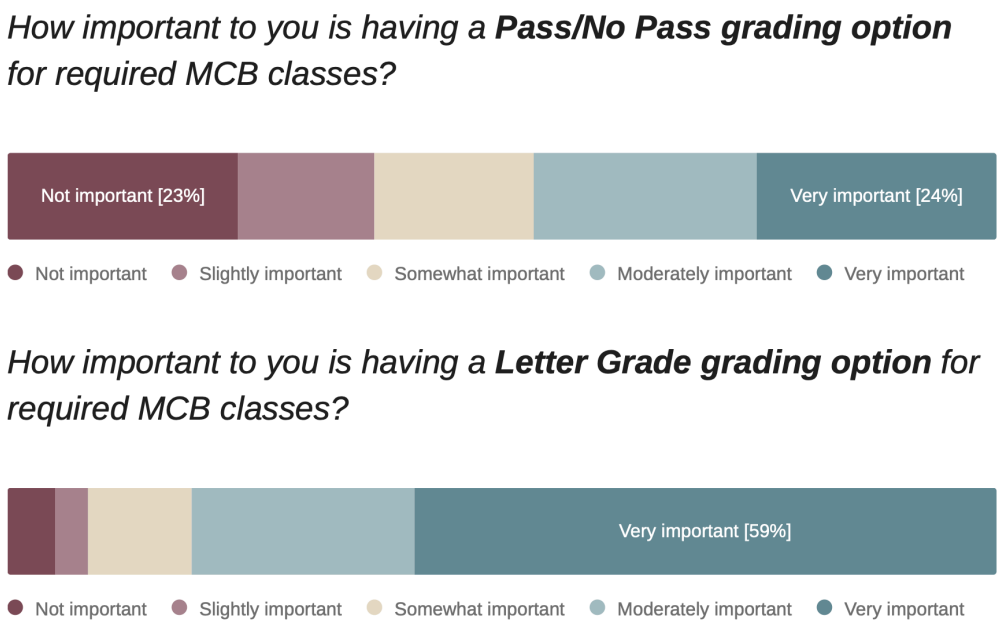


Figure 4.14: Students rated the importance of having a Pass/No Pass or Letter Grade option available for required MCB classes.

Students had varying opinions about the extent to which their MCB instructor(s) took steps to reduce cheating and make exams more equitable, with responses evenly spread from not at all to a great deal (Figure 4.15). Similarly, student responses were spread on whether they were concerned about cheating in Fall 2020 (Figure 4.15).

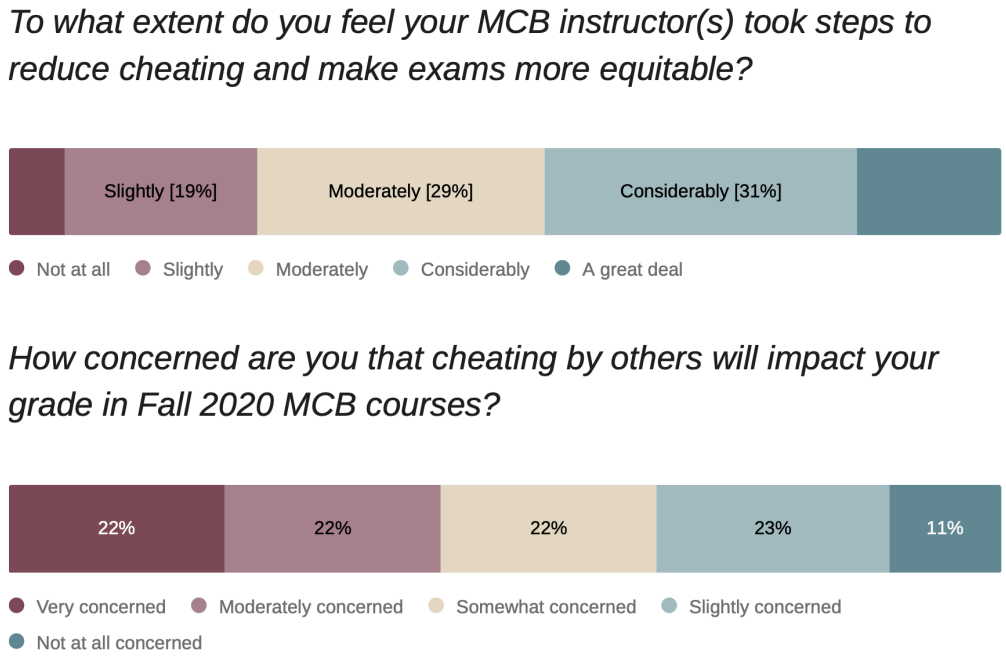


Figure 4.15: Students rated the extent to which MCB instructors took steps to reduce cheating and how concerned they were about cheating by others impacting their grade in Fall 2020 MCB courses.

Student comments and suggestions related to grades and exams are paraphrased as follows: 1) The formal decision on pass/no pass should be announced as soon as possible by the department and course instructors to avoid confusion, 2) Reduce the percent of grades coming from exams to reduce impact of cheating, 3) Provide quizzes and assignments throughout the semester to reduce anxiety and cheating, while ensuring students stay on top of the material, 4) Make exams open-note and open-book to reduce unfairness due to cheating. Student comments were diverse in terms of whether pass/no pass grading should be an option and the ideal exam procedure (some students advocated for flexibility to take exams within a 24-48 hour window to increase equity for students with technical difficulties or different time zones, some students advocated for time-limited exams to reduce cheating).

4.3.1.6 Demographic Data and Analysis

To examine whether our survey population was representative of the general MCB undergraduate population, we compared demographic data from our survey to MCB department-wide student data from CalAnswers (kindly provided by Katie Carson). Overall our survey population was similar to the general student population along the axes of identity for which general data was available: disability status, race/ethnicity, and gender.

11% of students in our survey reported having a long-lasting or chronic condition that substantially limits one or more major life activities, while 10% preferred not to answer this question. These numbers are higher than the 6% of students with DSP accommodations in the general MCB student population, but this is likely expected if not all disabilities require or receive DSP accommodations.

In terms of race and ethnicity, our survey had a slightly higher proportion of Asian students compared to the general MCB population (59% compared to 50%), a lower proportion of Hispanic/Latinx/Spanish origin students (5% compared to 13%), a similar proportion Pacific Islander students, and a similar proportion of White students. Our survey lacks any data from Black/African American students (0% compared to 2% in the general MCB population). Students identifying as American Indian or Alaska Native were not sampled in the general MCB population data, so we were unable to assess whether this group was appropriately represented in our survey.

Students identifying as female/woman/cis female were the majority of respondents (70%) which reflects the high percentage of students identifying as female in the general MCB population (62%). Students identifying as male were underrepresented in our survey (15% compared to 37%). Students with genders besides male and female were not sampled in the general MCB population data, so we were unable to assess whether additional genders were appropriately represented in our survey.

We also collected demographic information not available in the MCB general population data set. For sexual orientation, 75% of students identified as heterosexual, 7% as bisexual, 14% as other sexualities (gay or lesbian, fluid, pansexual, queer, demisexual, asexual, or questioning), and 4% preferred not to answer this question. 48% of respondents received any form of financial aid.

Most students (71%) expect to work during Fall 2020, including 61% working 16 hours or less per week and 10% working 17 hours or more. Most students had no primary caregiving responsibilities, though 4% of students will be the primary caregiver for dependents during Fall 2020, and 3% did not know yet if they would be primary caregiver.

Most students (81%) expected to be living in the Pacific Time Zone during Fall 2020 when our survey was in July, though 5% did not expect to be in the Pacific Time Zone and 14% didn't know yet.

4.3.2 Resources and Recommendations for Instructors Teaching Remotely

We performed a review of education research literature to create a summary of best practices for MCB instructors to use during remote learning during the COVID-19 pandemic. We particularly focused on literature relevant to undergraduate STEM education and online learning. We summarized our results as four main recommendations with supporting rationale (Why?), techniques for implementation (How?), and further resources. The recommendations were formatted

as a series of web pages on our departmental website for ease of distribution and navigation for instructors, with interactive links and drop-down menus (Figure 4.16, Figure 4.17).

Figure 4.16: Main page of "Resources for Teaching Remotely" website

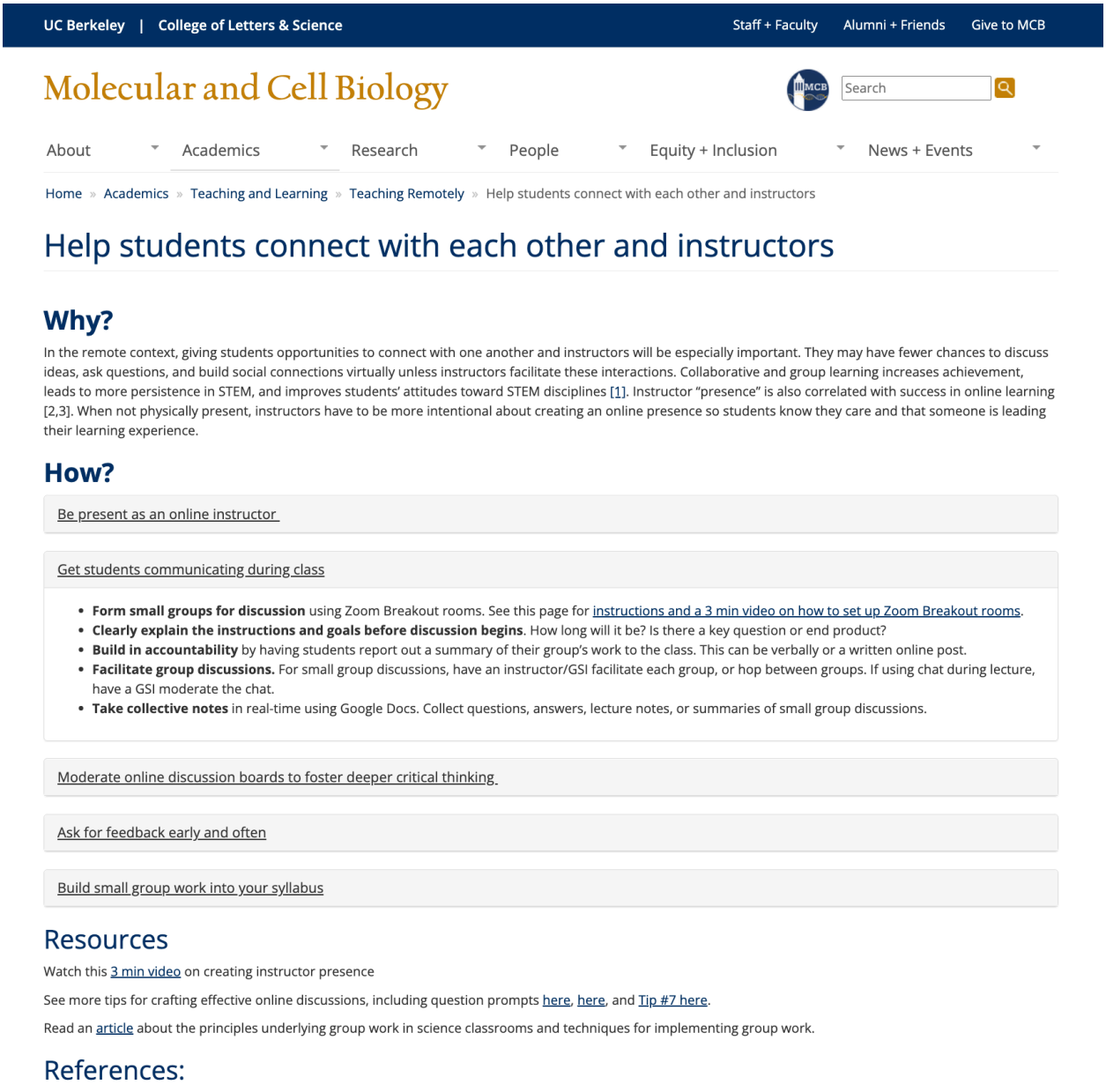


Figure 4.17: Example recommendation-level page of "Resources for Teaching Remotely" website. Rationale, techniques, resources, and references are included for the recommendation "Help students connect with each other and instructors"

The recommendations we developed, also available at the Teaching Remotely website, are reproduced in full below.

4.3.2.1 Create an Inclusive Online Learning Environment

Why?

Students have a wide variety of backgrounds, identities, and accessibility needs, many of which may not be visible. Students from underrepresented groups continue to be enrolled at lower rates at UC Berkeley than they are present in California's population and, when they do enroll, equity gaps in graduation rates persist (Fink et al., 2018). Thoughtful teaching practices and course design can help to close equity gaps and enable all students to thrive (Theobald et al., 2020).

How?

Establish a respectful and inclusive course culture

- Explicitly state course norms to create a positive and respectful learning environment
  - Encourage a growth mindset - the idea that ability is not innate, but can be developed - in your students and yourself in order to increase motivation and decrease achievement gaps (Fink et al., 2018; Canning et al., 2019).
  - Set the tone by establishing classroom ground rules and including a diversity statement in your syllabus.
  - Help students develop awareness of multiple visible and invisible identities in the classroom, including race, gender, disability, and nationality.
- Give students the opportunity to tell you their preferred name and pronouns before calling attendance or using pronouns in class. Require respectful screen names and encourage, but do not require, pronouns in screen names (Russell et al., 2018).
- Connect science to its social context by including examples of the diverse scientists behind the work, and by using inclusive language when discussing science related to gender, race, or disability.
- Address violations of virtual norms immediately. Microaggressions, which are slights against marginalized populations which may be unintentional, increase stress and cognitive load, and can come in many forms (Harrison and Tanner, 2018). Countering microaggressions with encouraging microaffirmations can increase persistence in science for marginalized groups (Estrada et al., 2019). Learn how to respond to tense, "hot moments".

Promote participation of all students (Tanner, 2013)

- Provide options for synchronous (in-class) and asynchronous participation, including anonymous participation (Latham and Hill, 2013). Not all students may be in a time zone that permits in-class participation, and among those that do, privacy or technology issues may make some students unable to share their video. Use a Piazza discussion forum for asynchronous participation.

- Encourage participation from all students - not just the most confident and immediate speakers - by increasing wait time after asking a question. Giving students even just 3-5 seconds to think improves class discourse and achievement (Tobin, 1987). Require (virtual) hand-raising, and consider waiting for multiple hands to give more students the option to participate - this can be done even in a large-enrollment class.

Make course materials accessible

- Check in with your students about their accessibility needs, technology access, and well-being. Note that you are required to provide accommodations that have been approved by the Disabled Students Program.
- Use accessible formats for course materials. PDF format is best suited to screen readers. Include image descriptions and alt-text for images, and captions for videos.
- Be mindful that English may not be a first language for some participants, including some international students.

Resources

- Watch a 3 minute video about growth mindset (The University of Arizona, 2017).
- Read an article on fostering trans inclusion in the classroom (5 minute read) or watch a short video on why pronouns matter (2 min) (As/Is, 2015; Grover, 2016).
- Check out some resources and tips for inclusive teaching in large courses (2 minute read) (Inclusive Teaching at U-M, n.d.).
- Look into twenty-one teaching strategies to promote student engagement and cultivate classroom equity (long read, but includes short list of actionable tips) (Tanner, 2013) .
- Determine your students' accessibility needs using this example survey from NYU (Glabau, n.d.).
- Read an article including more accessibility tips (5 minute read) (Hamraie, 2020).
- Brush up on faculty resources related to the UC Berkeley Disabled Students' Program (DSP) (UC Berkeley Disabled Students' Program, n.d.).

4.3.2.2 Provide Clear Course Materials Organized within a Structured Website

Why?

Without the structure of an in-person class and teacher, it is very easy for students to get lost and confused. Confusion about assignments, deadlines, and requirements is a major challenge for online students. Increasing course structure can result in higher grades for all students, but especially for student groups that are underrepresented in STEM (Eddy and Hogan, 2014).

How?

Provide a clear, well-organized syllabus that sets the tone and expectations for the semester (Miller and Tanner, 2015) . The syllabus should include the following information:

- A clear course description and clear course policies. Explain your policies and expectations for inclusive classroom behavior, academic misconduct, late work, grades, etc. Include a reminder for DSP students to contact you about accommodations.
- Instructors' modes of communication (ie. email, Zoom, Piazza), including the time frame students can expect to hear back from you (ie. 24 hours, within a week, weekends/after-hours policies, possibly different prior to major assessments).
- Learning outcomes. What knowledge/skills are expected as prerequisites for the course? What knowledge should students gain and what skills should they build by taking the course? Setting learning goals will help you assess whether your teaching is working and allow students to assess their progress (Petersen et al., 2020).

Have clear, consistent schedules and avenues of delivering information (Jaggars and Xu, 2016)

- Make an introductory video where you show students how to navigate bCourses (UC Berkeley's Canvas) and discuss how the class will be organized.
- Make extensive use of bCourses to deliver material and assignments. Keep files and assignments organized. Use the modules tool or pages to organize links, files, videos and assignments for one topic in one place.
- Make assignments available on a consistent basis (i.e., weekly or after lectures). Make modules available one at a time, to keep students from falling behind or feeling overwhelmed.
- Clearly organize concepts into a framework that provides students with the "big picture." Remind students of the learning goals for each module and how they fit into the learning goals of the class.

Communicate expectations and send reminders

- Provide rubrics and share examples of "model" work. Rubrics define the skills or knowledge students are expected to demonstrate for an assignment. Share exemplary 'model' assignments or discussion posts so students know what to strive for.
- Send reminder announcements about due dates or updates to plans for the week.

Resources

- Check out an example of a syllabus from CSU Channel Islands (California State University, Channel Islands, n.d.).
- Find more syllabus design tips from the Berkeley Center for Teaching and Learning (3 minute read, includes links to more detailed information on specific topics) (UC Berkeley Center for Teaching and Learning, n.d.b).
- Consider Principle #4: Organize Course Content Intuitively from the article "How to Be a Better Online Teacher" from The Chronicle of Higher Education (Darby, 2019).

- Find more tips for organizing your bCourses site from UC Berkeley's IT knowledge base (3 minute read, includes links to more detailed bCourses tutorials) (UC Berkeley IT Knowledge Base, n.d.).

4.3.2.3 Help Students Connect with Each Other and Instructors

Why?

In the remote context, giving students opportunities to connect with one another and instructors will be especially important. They may have fewer chances to discuss ideas, ask questions, and build social connections virtually unless instructors facilitate these interactions. Collaborative and group learning increases achievement, leads to more persistence in STEM, and improves students' attitudes toward STEM disciplines (Springer et al., 1999). Instructor "presence" is also correlated with success in online learning (Baker, 2010; Sheridan and Kelly, 2010). When not physically present, instructors have to be more intentional about creating an online presence so students know they care and that someone is leading their learning experience.

How?

Be present as an online instructor

- Communicate frequently to show students you are involved. Post weekly schedules or announcements, contribute to online discussions.
- Post an introductory video telling students about yourself and establishing your persona
- Host office hours that are accessible to students in different time zones or with different work schedules
- Bring your personality and real-world expertise to your class. As an instructor, you bring knowledge of what aspects of the material are often difficult to learn, how the topics of the course link together, and how the material relates to the broader world.
- Reach out to struggling students.

Get students communicating during class using Zoom Breakout rooms, chat, or Google Docs

- Form small groups for discussion using Zoom Breakout rooms.
- Clearly explain the instructions and goals before discussion begins. How long will it be? Is there a key question or end product?
- Build in accountability by having students report out a summary of their group's work to the class. This can be verbally or a written online post.
- Facilitate group discussions. For small group discussions, have an instructor/GSI facilitate each group, or hop between groups. If using chat during lecture, have a GSI moderate the chat.

- Take collective notes in real-time using Google Docs. Collect questions, answers, lecture notes, or summaries of small group discussions.

Moderate online discussion boards to foster deeper critical thinking and writing

- Piazza is preferred as an online discussion platform among MCB students (Arnold, Madeline and Beatty, P. Robert and Bormann, Alyssa and Moreno, Joseph and Navas, Kathleen and McSharry, Maria and Xu, Nicole, 2020). You can set up Piazza for your Berkeley course, and add Piazza to your bCourses page. The bCourses "Discussions" function is another option, but can be harder to navigate and track responses to particular topics. Specific discussion topics can be assigned to students in bCourses and graded quickly using SpeedGrader.
- Know your learning goals for the online discussion. Is it to build community? To provide Q&A? To encourage debate or scientific reasoning? See example discussion goals along with example prompts, responses, and rubrics for grading posts from Brown University (The Harriet W. Sheridan Center for Teaching and Learning, n.d.).
- Communicate clear expectations about participation to students. How often will they be expected to post? What are the community standards for inclusive discussion? Provide criteria for good discussion posts and highlight exemplary 'model' posts so students know what to strive for.
- Use open-ended questions with multiple "right" answers to get more productive discussions. See tips on writing questions and prompts for online discussions (Boettcher, n.d.; The Harriet W. Sheridan Center for Teaching and Learning, n.d.) .
- Check the board a few times a week. Post clarifying questions/responses and respond to "orphan" posts with no responses. Don't over-post if you want students to answer each other's questions.
- Consider assigning students as weekly discussion leaders. They can post questions, moderate, highlight key posts.

Ask for feedback early and often

- Get to know students by collecting basic info on name pronunciation, background, interests, location, and access to learning technology. If students don't have the tech they need, direct them to Berkeley Student Technology Services.
- Survey the class for immediate feedback using Zoom polls or informally in the Zoom chat. Other options include Kahoot, Poll Everywhere, and iClickers.
- Get feedback after each class using formative assessments like the minute paper or muddiest point (formative evaluations check understanding during the learning process so instructors can adjust their teaching) (UC Berkeley Center for Teaching and Learning, n.d.a).
- Respond to midterm evaluations. By directly addressing student feedback, you demonstrate your investment in their learning. If you have access, re-read evaluations from previous years before the semester starts to incorporate feedback even earlier.

Build small group work into your syllabus

- Jigsaw activity in class or lab. This is a cooperative learning activity where students are assigned a specific topic to research before class (e.g. an article), then are assigned to teams with students that have researched different material so they can teach one another (Schreyer Institute for Teaching Excellence, Penn State, 2007).
- Assign group problem sets, quizzes, or reports. Assigning group roles (like Facilitator, Recorder, Reporter) can be effective. Consider keeping groups with consistent members for a period of time to foster positive relationships/group dynamics (Center for Teaching and Learning, Washington University in St. Louis, 2020).
- Incorporate peer-review of assignments or writing
- Have a plan to assess and address any problematic group dynamics [5]. In long-term group projects students can send weekly reports to the instructor noting their progress, learning, and issues, which can help the instructors know if intervention is necessary.

Resources

- Watch this 3 min video on creating instructor presence (Quality by Design, 2016).
- See more tips for crafting effective online discussions, including question prompts (Massachusetts General Hospital Institute of Health Professions, 2016; Simon, 2018)
- Read an article about the principles underlying group work in science classrooms and techniques for implementing group work (Tanner et al., 2003).

4.3.2.4 Develop Effective and Fair Assessments

Why?

Assessment can serve many purposes, such as measuring mastery, ranking students, communicating feedback, motivating students, or rewarding effort (Schinske and Tanner, 2014). Fair grading practices ensure that grades provide meaningful information to instructors and students. What's more, assessments don't just determine a student's final grade, they can enhance learning and allow you to monitor class understanding of difficult concepts (Roediger and Karpicke, 2006; Brame and Biel, 2015; Xu et al., 2017).

The way you grade will affect how students learn and behave; for example, grading on a curve may promote competition since success of one student comes at the expense of their peers, whereas mastery-based grading may encourage collaboration since the success of one student is not contingent on the performance of their peers.

How?

Clearly state how grades will be determined in your course.

- Develop a fair grading system from the get-go. Different grading schema are available (Eberly Center, n.d.b)
- Be transparent with students about how they will be graded. Ambiguity increases cognitive load. If students do not know what is expected of them, they may underperform for reasons unrelated to their level of knowledge or skill.

Use frequent assessment to promote and measure learning

- Assess your students frequently. Assessment promotes learning and provides data about what students have and haven't learned (Roediger and Karpicke, 2006; Brame and Biel, 2015; Xu et al., 2017). Assessment can be graded (e.g. quizzes, exams) or ungraded (e.g. polls, practice worksheets) assessments.
- Incorporate low-stakes assessments (e.g. small quizzes) when grading in addition to high-stakes testing (e.g. exams). Relying only on a few high-stakes assessments can amplify the effects of having a bad day on a student's final course grade .

Develop questions that test the most important concepts or skills.

- Clarify desired learning outcomes ahead of time.
- Teach what you will test, and test what you have taught (Eberly Center, n.d.a).
- Grade only what you are trying to measure. Avoid questions and grading schemes that test variables unrelated to what you are trying to measure. For example: deducting points for minor spelling errors in an essay question about the central dogma means that the grade is partially a measure of spelling, not just knowledge of the central dogma .

Determine which platforms are best suited to the kind of assessments you want to do.

- Instant "clicker"/polling software such as Kahoot! or Poll Everywhere, allows collection of real-time data on students' understanding and encourages the entire class to participate. However, introducing additional platforms that students and teachers must juggle can cause confusion.
- bCourses.
  - Quizzes are fully integrated into the course, and may be easier for students to keep track of. On the other hand, they are harder to grade than Gradescope assessments.
  - Speedgrader is built into bCourses and can be used to automatically grade multiple choice and other types of questions, but can be frustrating and slow for grading large numbers of exams. It also uses specific scoring methods that may not be what the instructor intended; these can be modified by implementing scripts developed for this purpose, but this takes substantial time and effort (the scripts must be downloaded from Github, installed, and often tweaked to work properly with bCourses).

- Gradescope exams and quizzes are easiest to grade quickly, especially for large exams and when multiple graders are working together. There is a slight learning curve for students and teachers to use this platform. Give students a timed practice assignment on Gradescope before giving your first timed online assessment, so they can practice using the platform.

Resources

- Determine how well your assessments align with your goals using this assessment evaluation rubric (long read, but the rubric itself is provided in the appendix at the end) (Tractenberg, 2020).
- Watch a short video on using Kahoot! as "clicker"-type software (a few 1-2 minute videos) (Kahoot!, 2020).
- Watch a video on creating and managing bCourses Quizzes (4 minute video) (Instructure Community, 2015).
- Learn how to use Gradescope to create and grade exams and assignments (short videos for different assignment types, each 2-5 minutes long) (Gradescope, n.d.).

4.4 Discussion and Recommendations

The results of our student experiences survey suggest that overall, many students were negatively impacted by remote learning. Students reported difficulties including distractions/noise, increased home duties, work shifts overlapping with class, tech issues, and time zone issues, with some students experiencing multiple difficulties at once. Unreliable internet access was a common concern, although most students reported reliable access to technology. However, since this survey was administered online, it is likely not capturing MCB students with severe limitations in technology or internet access. Students reported increased need for mental health care, financial assistance, and other services. These data suggest that interventions that address barriers to learning and provide for student needs are required to successfully respond to acute crises like the global pandemic in 2020 and transition to remote learning.

Through both quantitative and qualitative responses, students provided feedback about their preferences for online learning in MCB courses. To best promote learning and engagement with course material, students recommended live lectures and accessible office hours. Office hours and study groups were rated as engaging for most students, but many students did not participate in these activities or reported office hours as only somewhat accessible to them. These data suggest that there is untapped potential for increasing student engagement in remote courses through participation in office hours and study groups. Instructors could boost participation in office hours by increasing access (frequency, time, method). These results are aligned with results from our literature review, which also emphasized the importance of student-instructor

and student-student interaction time as an important component of learning, especially online learning.

MCB student feedback and best practices from the literature indicate there are likely ways to increase student engagement and interaction using methods and technologies specific to the online environment. To facilitate interaction outside the classroom, students predominantly preferred an instructor-monitored Piazza over any other method. This asynchronous option for discussion between students and with instructors could be more widely implemented in MCB courses. To support interaction and engagement during class time, students indicated that they appreciate when a second instructor monitors a live chat to answer questions. There were mixed reviews regarding the effectiveness of Breakout rooms, with students agreeing that Breakout rooms need to be structured in order to effectively promote conversation. We found similar results in our literature review, with scholars and practitioners emphasizing the importance of advance planning, clear communication, and accountability for successful student discussions. Many of these pedagogical interventions are relatively simple to implement, and could have a positive impact on student learning in remote courses.

Given the number of students who reported difficulty accessing a reliable internet connection, we recommend that instructors teaching remotely provide an asynchronous option. We also encourage instructors to consider accessibility by disabled students when modifying or designing course materials for remote instruction, and proactively check in with students about their access needs.

Both the education literature and student concern about exams suggest that courses would likely benefit from re-thinking the practice of few, closed-note, high-stakes exams. Instead, instructors can incorporate changes such as decreasing the portion of grades that come from high-stakes exams, writing exam questions that are compatible with an open-note format, or finding alternative forms of assessment to exams (e.g. projects, papers, reports, presentations). We also suggest implementing and clearly communicating policies to reduce cheating and its impacts (e.g. grading based on competency rather than comparison between students).

4.4.1 Recommendations to MCB Department Leadership

In our survey of student experiences and when comparing best practices in STEM education to current practices in the MCB department, we observed structural issues beyond what individual instructors may be able to address. In order to begin to tackle some of these larger issues, we prepared the following eight simple recommendations to MCB departmental leadership in Fall 2020 for implementation during remote learning:

1. Provide trainings and information for undergrads on study skills and creating inclusive virtual classrooms.
2. Raise funds for an undergrad student relief fund.
3. Hire additional GSIs if necessary to provide frequent office hours that are accessible from various time zones.

4. Continue collecting feedback from students on their experience and needs.
5. Provide technology to students (WiFi hotspots, computers) and instructors (microphones).
6. Promote course structures with several small low-stakes assessments rather than few high-stakes assessments, and encourage open-note exams.
7. Encourage instructors to have clear and flexible deadlines and grading policies, including attendance.
8. Train instructors to recognize and support struggling students.

4.4.2 Conclusions and Applicability Beyond the COVID-19 Pandemic

Many MCB undergraduate students reported experiencing significant stressors in 2020 that likely detracted from their education. These results highlight the importance of instructional flexibility and attention toward students needs during economic and health crises like the global pandemic that led to the transition to remote learning in Spring 2020. However, students experience similar stressors even outside a pandemic context. Student needs should be evaluated on a constant basis at both the course and department level, and educational policies that provide necessary services be continuously implemented and evaluated. Incorporating such a cycle of student feedback, policy implementation, and program evaluation into the MCB program would allow for increased resilience in the face of future crises, since frameworks to quickly assess and address needs would be in place.

Many of the interventions discussed here likely apply beyond remote courses to in-person or hybrid courses as well. Having a recorded lecture option could provide more learning flexibility for students with health issues, disabilities, and work or home responsibilities. Increasing student participation and engagement during class time, and outside class through office hours, study groups, and discussion boards, could increase equity and overall learning outcomes. Reformatting curricula and grading policies to reduce the proportion of grades from high-stakes exams and incorporating alternative forms of assessment could improve the accuracy and equity of assessment, and thereby improve access to STEM careers.

5 "Building A Sense of Belonging" Inclusive MCB Conference Assessment

5.1 Introduction

5.1.1 Toward Equity for Under-represented Groups in STEM: Institutional Change and Sense of Belonging

When entering college, under-represented minority (URM) and non-URM students plan to major in STEM fields at the same rate (Olson and Riordan, 2012). However, when examining outcomes at progressively advanced stages of the traditional STEM education and career path, rates of URM retention decrease, and few URM scientists hold leadership positions (Leboy and Madden, 2012; Estrada et al., 2016; National Center for Science and Engineering Statistics, 2021). This outcome has come to be known as the "leaky pipeline," and though this metaphor improperly suggests a linear STEM career path, it demonstrates the inequitable outcomes produced by the scientific enterprise (Batchelor et al., 2021).

Lower retention of URM scientists is a symptom of a larger system of discrimination in science. Scientific fields were built within oppressive systems such as colonization, racism, and sexism, and scientific institutions continue to perpetuate these historical power structures. STEM fields display discrimination that manifests at multiple levels, including ignorance of discrimination and privilege, epidemiological monopoly by Western knowledge systems, hostile academic environments, harassment, biases in hiring and collaborations, and disparities in grant funding and compensation (Cech et al., 2017; Salerno et al., 2020; Diele-Viegas et al., 2021; National Center for Science and Engineering Statistics, 2021; Taffe and Gilpin, 2021; Chen et al., 2022).

In developing solutions to these entrenched problems, research and activism have shifted from theories that situate issues and interventions at the individual level (e.g. imposter syndrome) to systemic approaches (Clance and Imes, 1978; Liu, 2018; Tulshyan and Burey, 2021; Fischer, n.d.). Work in the field of organizational management has developed frameworks for the process of institutional change to advance equity. Studying gender equity in academic STEM, Bilimoria and Singer (2019) has defined frameworks for institutional transformation, including facilitating factors, initiatives, research and evaluation, institutionalization, and outcomes, as well as a model of effective practices for transformation at the individual, department, and university levels. Proposed institutional practices to address the failure to retain URM scientists include pedagogical

reform, research support programs and STEM learning communities, mentorship and faculty accountability, and creating a culture of macro and micro affirmations (Whittaker and Montgomery, 2013; Estrada et al., 2018).

At the department and community level, we can begin to address systemic exclusion of scientists from marginalized groups and increase diversity in science by building a culture that promotes a sense of belonging for all members. Sense of belonging is important to whether or not students remain in STEM majors, and women and students of color, particularly women of color, are less likely to feel they belong in STEM (Johnson, 2012; Strayhorn, 2012; Rainey et al., 2018). Peer relationships and support can increase feelings of belonging, and a psychological intervention that mitigated doubt about social belonging led to academic benefits for Black students (Walton and Cohen, 2007; Espinosa, 2011; Johnson, 2012). Therefore, community interventions that bolster peer and mentoring relationships and reduce feelings of doubt about belonging have the potential to increase the number of under-represented students who can remain and thrive in STEM.

5.1.2 Research and Evaluation of Equity Initiatives

When implementing practices and policies to promote diversity and equity in science, research and evaluation can be applied to measure these initiatives' success. Cycles of design, evaluation, and redesign of initiatives are an important component of transformational change within an institution. Program assessment can check whether programs are meeting their stated goals, investigate the mechanisms behind positive or negative outcomes, incorporate feedback from stakeholders, and adapt the program to new contexts. Evaluation can also legitimize programs and direct funding to effective interventions. Often long-standing, successful initiatives like the Biology Scholars Program at UC Berkeley can be passed over for funding in favor of new, untested initiatives (Sanchez, 2021). Using a data-driven, evidence-based approach to implementing programs can help ensure new programs work and successful programs continue. Importantly, evaluation efforts need to be knowledgeable and attuned to the needs of the community being studied, aka culturally competent (Mertens and Hopson, 2006).

5.1.3 Inclusive MCB (iMCB) Initiative at UC Berkeley

Dr. Lisa Eshun-Wilson, then a graduate student in UC Berkeley's Molecular and Cell Biology Department (MCB), founded the Inclusive MCB (iMCB) initiative in 2018. Her vision was to increase the sense of belonging and alleviate the "minority tax" (e.g. disproportionate time and energy spent on service work) on marginalized scientists (Rodríguez et al., 2015; Gewin, 2020; Williamson et al., 2021). The initiative was designed as a space to discuss issues disproportionately impacting historically underrepresented students and advocate for sustainable, evidence-based solutions. iMCB grew into a graduate student and postdoc led organization running multiple programs for biosciences trainees including a faculty mentorship program, an annual conference, postdoc teaching and mentorship program, and affinity groups. These efforts were supported by

a leadership team (student directors Lisa Eshun-Wilson and later Michelle Soto Reid and Kyle Tucker), a research group to review literature and provide recommendations for program design, and an assessment team to evaluate the impact of programs.

The annual conferences designed and coordinated by iMCB began in 2018 and were incorporated into the first-year graduate student orientation for multiple UC Berkeley departments including MCB, Helen Wills Neuroscience Institute, and Computational Biology. Each conference was designed around a theme of particular relevance to scientists from historically underrepresented groups, such as "Bridging the Gap" between traditional academia and personal authenticity, "Building a Sense of Belonging" in science, and "Strengthening Your Roots and Growing Together" to strengthen ties to personal background and identity through community. Conferences featured talks from scholars of belonging and mentorship in STEM, as well as under-represented scientists sharing their personal stories. Students and faculty could attend workshops run by UC Berkeley's Multicultural Education Program, as well as affinity groups to meet other scientists with shared identities (including racial, ethnic, class, gender, sexuality, or family structure identities).

5.1.4 "Building a Sense of Belonging" iMCB Conference

The following section focuses on data from the Fall 2020 "Building a Sense of Belonging" iMCB Conference. I helped to organize and assess the conference as part of the iMCB Conference Team alongside Michael Ly, Hannah Nilsson, and Tram Nguyen, and as part of the iMCB Assessment Team along with Hannah Weaver, Michael Ly, and Mark Stepaniak. Michelle Reid and Kyle Tucker, the iMCB co-directors at the time, also contributed hugely to the conference organizing and provided overall guidance. This conference was held remotely over Zoom, and included 2 keynote speakers, 34 different affinity group discussions open to students and postdocs, workshops for first-years and faculty, and a first-year community hour.

We collected pre- and post-surveys to better understand the impact of the conference on the sense of belonging among attendees, as well as determine which events were most impactful to guide future conference planning. Our research questions for the surveys were as follows:

Questions relevant to both pre-and post-survey :

- What is the sense of belonging of attendees? Does their sense of belonging change after the conference?
- Are sense of belonging and experiences of the conference different between URM and non-URM trainees?
- What is the impact of the conference on attendees' comfort sharing about their values and identities?
- Does the conference impact attendees' interest in participating in diversity, equity, and inclusion efforts?

Questions relevant to pre-survey:

- What is the student's outlook as it relates to the MCB community when starting at UC Berkeley?
- What are attendees' expectations of the conference?

Questions relevant to post-survey:

- Which events were most impactful?
- What feedback on the conference do attendees have?

We found that many of the events were impactful, particularly for under-represented minority attendees, and we also observed increases in belonging post-conference.

5.2 Methods

The iMCB Assessment Team, made up of 4-6 graduate student members, evaluated the impact of iMCB projects and programs. Our main assessment instruments were surveys, and when possible we distributed both pre- and post-surveys for comparison of participant responses before and after an intervention. For each survey we prepared a document with overall survey goals, survey questions, and justifications for each question. The survey document was reviewed by all Assessment Team members as well as iMCB members outside the Assessment Team and a faculty mentor in order to incorporate feedback from as wide a range of perspectives as possible. We distributed surveys using Qualtrics. Due to privacy concerns and to solicit unhindered feedback, we implemented anonymous surveys. Therefore, pre- and post- surveys did not directly compare paired responses within one individual, but rather examined differences at the population level.

To assess the Fall 2020 "Building a Sense of Belonging" iMCB Conference, our team prepared surveys to measure respondents' sense of belonging and comfort sharing about identities before and after the conference, and their rating of the impact of each event. We investigated belonging in STEM and scientific identity by sourcing validated measures from the work of Dr. Mica Estrada (Estrada et al., 2011). We asked respondents to rate the extent they agree with the following statements: 1) I have a strong sense of belonging to the community of scientists, 2) I have come to think of myself as a 'scientist', 3) I feel like I belong in the field of science, and 4) Being a scientist is an important reflection of who I am. We asked respondents to rate how comfortable they are sharing their values and identities (e.g. ethnicity, gender, socioeconomic background) 1) with peers (all respondents), 2) with faculty (faculty excluded), 3) with trainees (faculty only). We also asked how much issues of diversity and inclusion factored into first year's decision to attend UC Berkeley, and to what extent all respondents planned to be involved in diversity, equity, and inclusion efforts in the coming year.

To learn about potential differences in sense of belonging and conference experiences between attendees from over and under-represented groups, we asked respondents whether they identified as an individual of an underrepresented minority (URM) group in STEM as defined by the NIH (NIH Chief Officer for Scientific Workforce Diversity, 2019). The NIH definition includes Black, Latino, and Indigenous people, people with disabilities, and people from disadvantaged backgrounds (including people who experience/experienced homelessness, foster care, were were eligible for Pell Grants, are first in their family to attend college, or grew up in a rural area). This question enabled us to stratify our survey data by whether respondents identified as URM to assess whether event impact or sense of belonging was different by URM status.

Distributions of responses between two groups (e.g. pre-survey and post-survey) were compared using the Mann-Whitney U test and associated p-values are reported (Mann and Whitney, 1947). Plots were generated using the ggplot2 package (v3.4.0) in R (v4.2.1), and annotated using Adobe Illustrator (Wickham et al., 2019; R Core Team, 2022). In plotting distributions, neutral responses were removed for diverging scales to ease interpretation of figures. The "Neutral" responses were removed for disagree/agree scales and the "Neither uncomfortable nor comfortable" responses were removed for uncomfortable/comfortable scales.

5.3 Results

We received 185 responses to the pre-conference survey and 147 responses to the post-conference survey, out of 217 people who attended at least one conference event (85% and 68% response rate to pre- and post-survey, respectively). Attendees represented 12 departments and graduate programs, across different positions (approximately 25% first-year student, 34% second-fourth year student, 11% fifth year and above student, 3% staff, 10% postdoc, 17% faculty). 35% of respondents identified as an individual of an underrepresented group in STEM in both the pre- and post-survey.

We found that for 32% of first-years, issues regarding diversity and inclusion were major or deciding factors in their decision when choosing a graduate program. For another 48%, these issues were a moderate factor, for 14% they were a minor factor, and for only 7% were they not a factor.

5.3.1 Keynotes and Affinity Groups were Most Impactful

Most participants found all events moderately or very impactful, with the most impactful events being the two keynote addresses and affinity groups (Figure 5.1). When separating responses by whether respondents identified as an under-represented minority in STEM (URM) or not (Non-URM), we found that all events except the first year community hour were rated as more impactful by URM compared to Non-URM. Affinity groups were rated significantly more impactful by URM (Mann-Whitney U test, $p = 0.014$) (Figure 5.2).

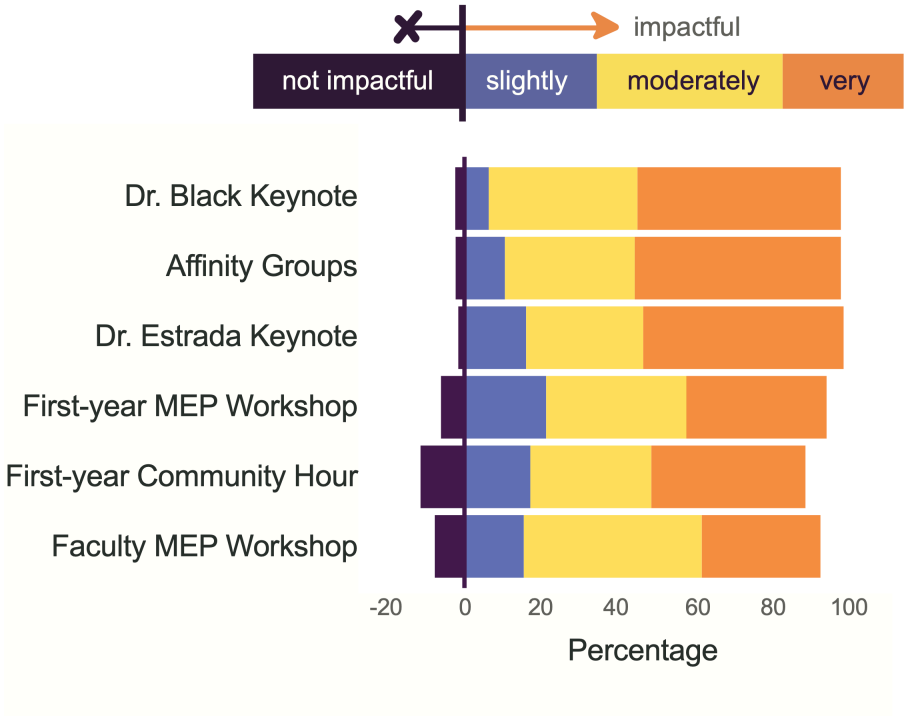


Figure 5.1: Attendee responses to the question, "Rate the following iMCB Conference events based on how much of a positive impact they had on you?" (n=148)

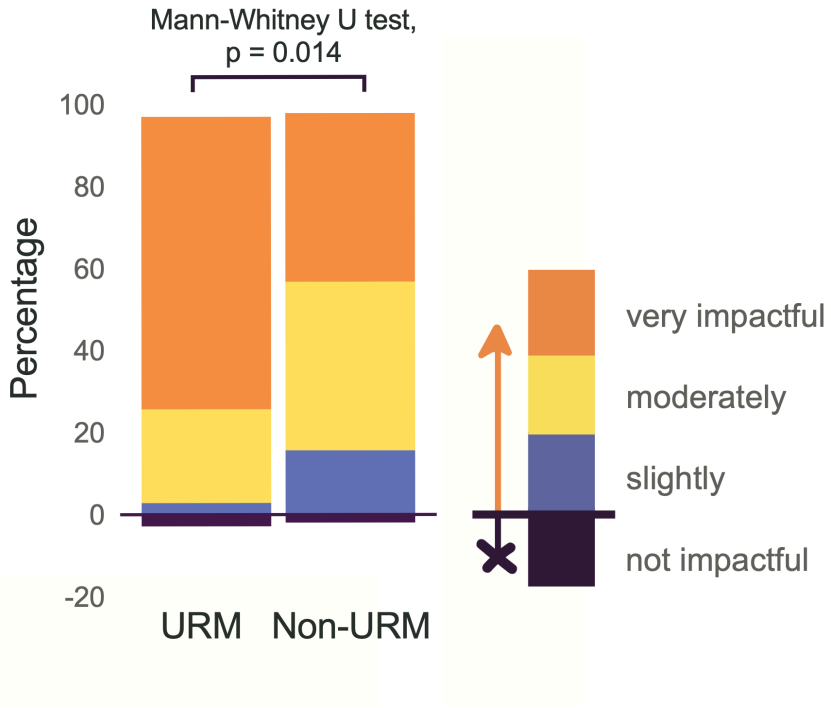


Figure 5.2: Affinity groups were rated as having a larger positive impact by respondents from under-represented minority groups (URM, n = 51) compared to respondents not from under-represented minority groups (Non-URM, n = 95).

5.3.2 Increased Sense of Belonging and Comfort Sharing about Values and Identities

We observed differences in attendees sense of belonging and comfort sharing about their identities and values after the conference. When examining the extent to which respondents agreed with the statement "I have a strong sense of belonging to the community of scientists," we found significantly increased agreement in the post-survey compared to the pre-survey (Mann-Whitney U test, p = 0.030) (Figure 5.3). When stratifying responses by URM status, we found trends toward increased belonging for non-URM compared to URM groups in both the pre- and post-survey, as well as a trend toward increased belonging for non-URM post-conference compared to pre-conference (Mann-Whitney U tests, p = 0.054, p = 0.054, p = 0.051) (Figure 5.4).

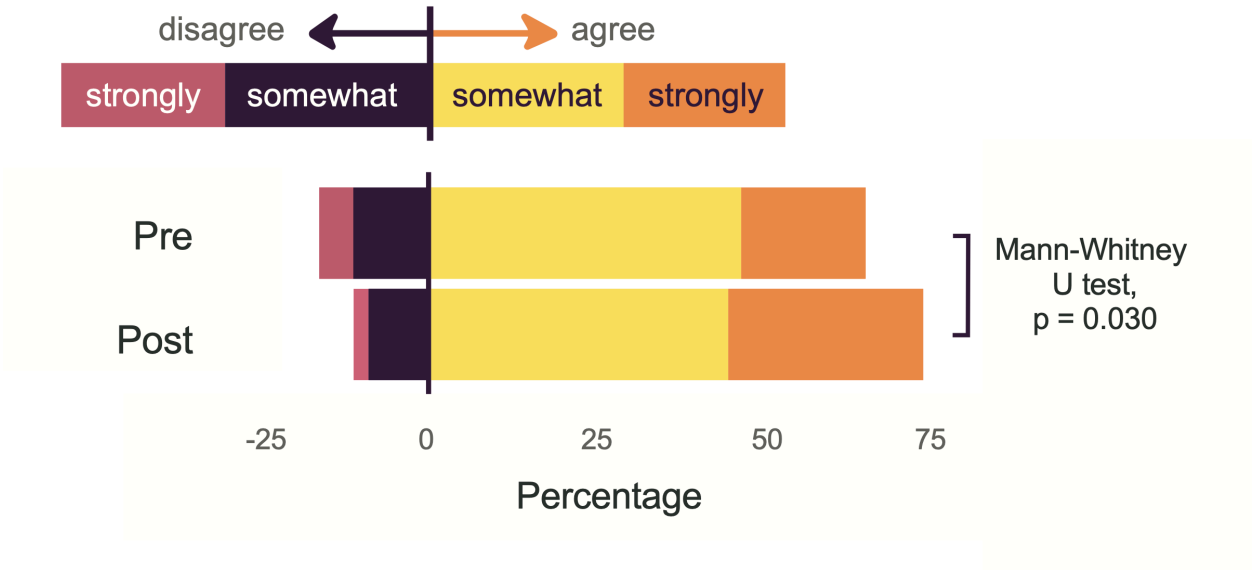


Figure 5.3: Attendees rated how much they agreed or disagreed with the statement, "I have a strong sense of belonging to the community of scientists." Data from pre- and post-surveys are shown. There was a significant difference in sense of belonging between the pre- and post-survey responses (Mann-Whitney U test, p = 0.030, pre-survey n = 179, post-survey n = 135).

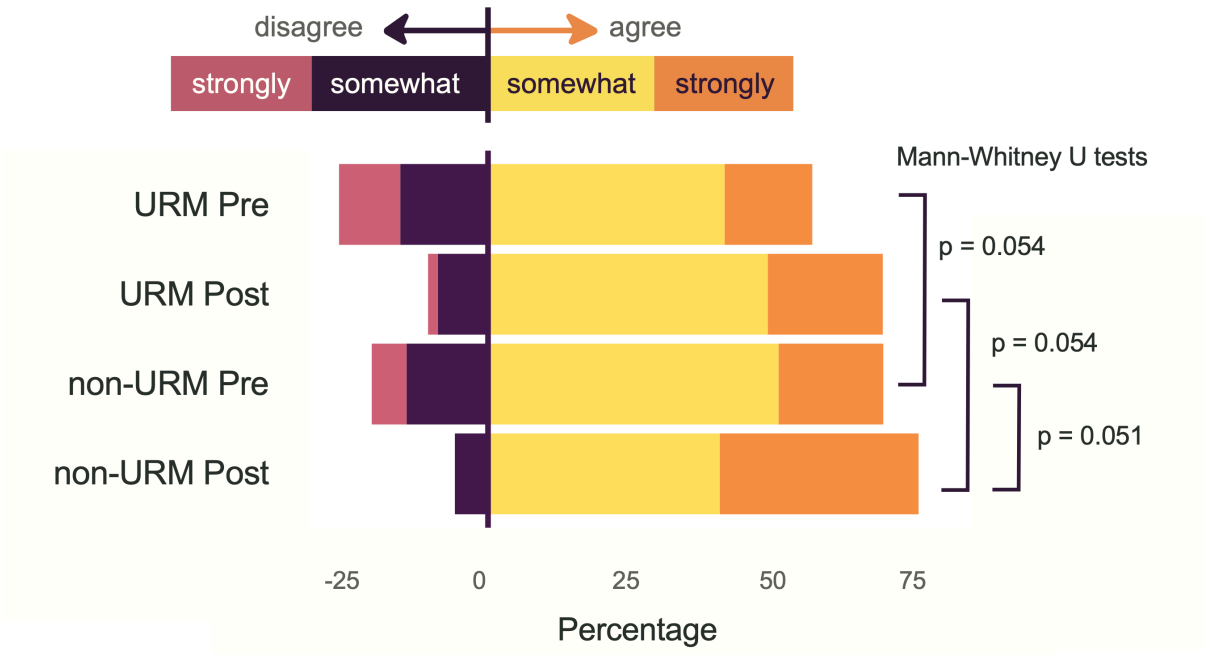


Figure 5.4: Attendees rated how much they agreed or disagreed with the statement, "I have a strong sense of belonging to the community of scientists." Data from pre- and post-surveys are shown, stratified by whether respondents identified as being from under-represented minority groups (URM) or not (Non-URM). Number of respondents were as follows: pre-survey URM, n = 65; pre-survey Non-URM, n = 114; post-survey URM, n = 49; post-survey Non-URM, n = 86. All reported p-values are from Mann-Whitney U tests.

We observed a significant difference in how comfortable respondents felt sharing their values and identities with peers after the conference (Mann-Whitney U test, p = 0.031) (Figure 5.5). We did not find a significant difference when looking at pre-post comfort levels among the URM group, though we did see a difference in comfort sharing values and identities among the non-URM group after the conference compared to before the conference (Mann-Whitney U test, p = 0.024, data not shown).

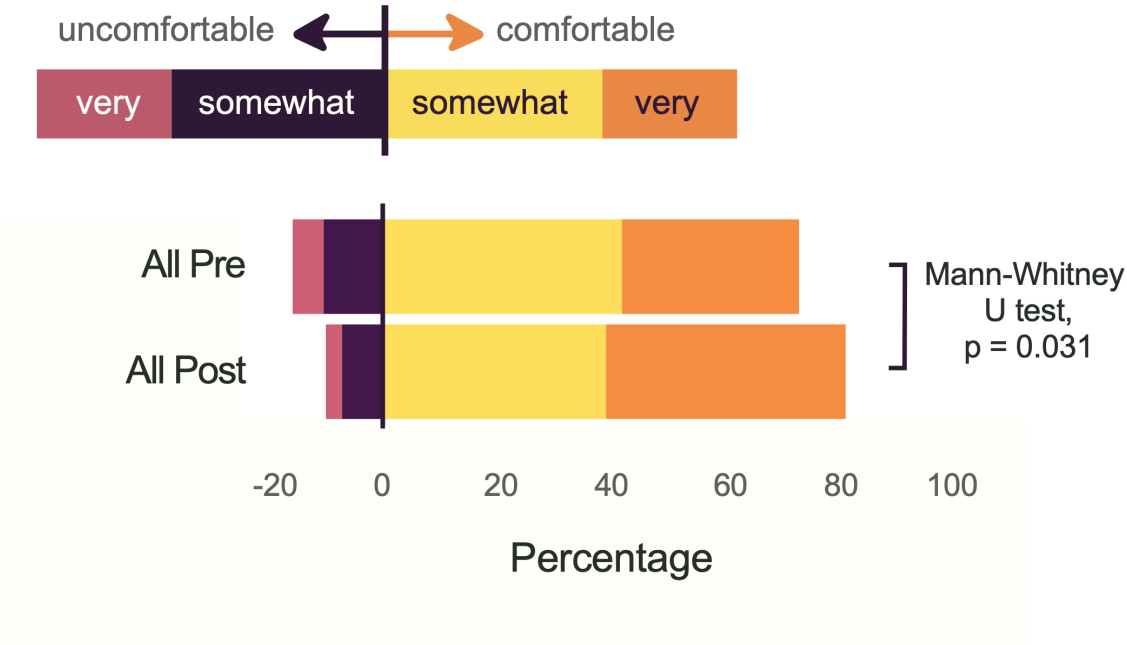


Figure 5.5: Attendees rated how comfortable they were sharing their values and identities with peers. Data from all pre- and post- surveys are shown, as well as the subset of data from first-year graduate students. There was a significant difference in comfort level reported in the pre- and post-surveys (Mann-Whitney U test, p = 0.031, pre-survey n = 183, post-survey n = 140).

5.3.3 Increased Plans to be Involved in Equity Efforts

We asked attendees to respond to the question "To what extent do you plan to be involved in justice, equity, diversity, and inclusion work in the coming year (at Berkeley or the broader community)?" (Figure 5.6). We found a significant difference between the pre- and post- survey distribution of responses (Mann-Whitney U test, p = 0.010).

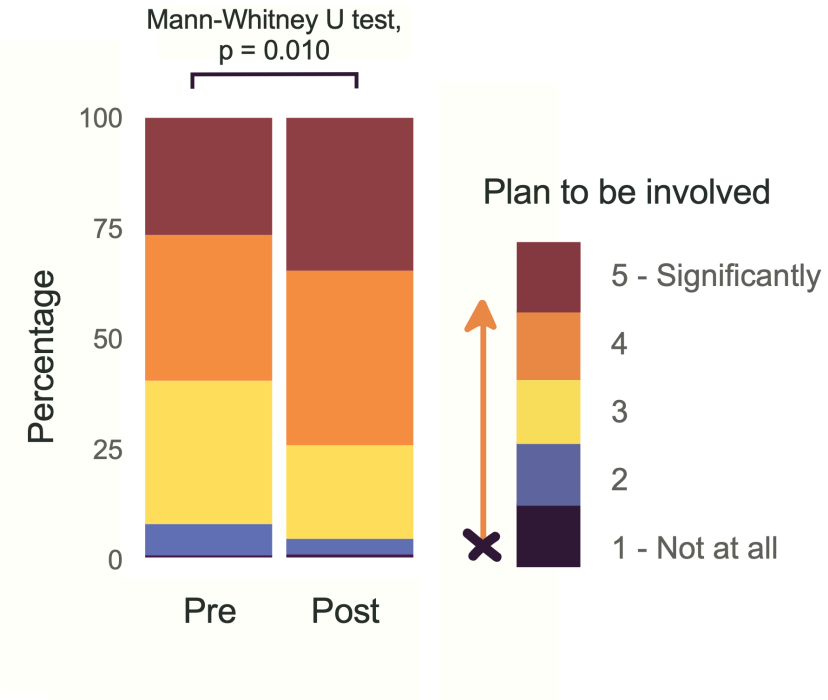


Figure 5.6: Attendees rated the extent to which they planned to be involved in justice, equity, diversity, and inclusion work in the coming year (at Berkeley or the broader community). There was a significant difference in planned involvement reported in the pre- and post-surveys (Mann-Whitney U test, p = 0.010, pre-survey n=184, post-survey n=141).

5.4 Discussion

5.4.1 Impact of the Conference

Our results show a clear positive impact of the Fall 2020 iMCB conference, for attendees from both under-represented and over-represented groups. Respondents found most events impactful, with affinity groups being particularly impactful for URM attendees. The fact that affinity groups had a particularly positive impact for people from under-represented backgrounds suggests that we succeeded in creating a space where people with shared identities could create community that may be lacking in everyday scientific and work spaces. These encouraging data led future conference organizers to continue affinity groups as part of iMCB and iBio conferences at UC Berkeley in 2021 and 2022, as well as the creation of a new iMCB 8-week summer affinity groups program which ran in 2021. For the question on event impact, only the affinity groups had a statistically significant difference in rated impact between URM and non-URM groups. These results suggest that the other conference events are similarly impacting a broad audience.

A key goal for the conference was increasing the sense of belonging among attendees, particularly those from marginalized groups, as well as increasing the capacity of individuals and our community to collectively work toward increased belonging for all. We observed an increase in sense of belonging reported after the conference compared to before the conference. The change in the distribution of responses was driven largely by fewer people reporting disagreement (5% to 2% strongly disagree, 19 to 16% somewhat disagree) and more people reporting strong agreement (18% to 29%) with the statement "I have a strong sense of belonging to the community of scientists" in the post-conference survey. We didn't see a shift in sense of belonging from before to after the conference when looking specifically at respondents identifying as URM. However, the sample size for URM respondents was smaller (n = 49 URM compared to n = 135 total in post-survey), reducing power to detect differences. We did see a trend toward increased sense of belonging in the non-URM group after the conference, indicating that our conference benefited non-URM attendees (Mann-Whitney U test, p = 0.051). Unfortunately, the trend of a gap in sense of belonging between URM and non-URM attendees before the conference (Mann-Whitney U test, p = 0.054) was still present after the conference (Mann-Whitney U test, p = 0.054). These data suggest that our conference increased sense of belonging for all attendees, but lower overall sense of belonging still remained for URM compared to non-URM groups after the conference. Given the long-standing exclusion of URM from academia and STEM fields, it is not surprising that gaps in sense of belonging are still present after the single day conference. However, by implementing targeted programs such as affinity groups for under-represented minority identities and keynote speakers with messages particularly relevant for these groups, we may begin to address this gap in belonging.

Another goal of the conference was to increase attendees' comfort sharing their values and identities with their scientific community. Perceived difference between an individual's values and the values of their field can lead to failures in STEM retention (Diekman et al., 2011). For example, in computing and engineering, perception that the disciplines aren't aligned with the value of communal goals like collaboration and helping others has deterred people who value communal goals from staying in the discipline (Boucher et al., 2017). By providing a space to discuss values and identities during the conference, our hope was to normalize values and identities that may not be considered typical in stereotypical ideas about STEM fields, and increase the comfort of attendees in continuing the conversation around their values and identities. Happily, we observed a significant increase in attendees' comfort sharing their identities and values with peers after the conference. We didn't see significant pre- vs post-survey differences when looking at first-years or faculty only (again, small sample sizes impact power in these cases). Interestingly, faculty who identified as URM seemed more comfortable sharing about their values and identities with trainees, only responding as somewhat or very comfortable, whereas 20% of non-URM faculty were either somewhat or very uncomfortable, and another 8% were neither uncomfortable nor comfortable. These data suggest that increasing the comfort of non-URM faculty in discussing their values and identities with trainees could be a potential mechanism for improving mentoring relationships for both URM and non-URM trainees with non-URM mentors.

Our survey results indicated that the conference inspired attendees to be more committed to participating in justice, equity, diversity, and inclusion work in the future. Given the limited impact

a single day of programming can have to improve the experiences of scientists, we were excited to see a shift in plans to participate in future efforts toward equity. Continuing commitment from community members will be required to sustain the increased sense of belonging and comfort sharing about values and identities that we saw immediately following the conference.

5.4.2 Limitations

One caveat to our survey data is the absence of a paired design. To maintain respondent anonymity and ease of taking the survey, we did not directly compare an individual's responses before and after the conference, rather, we compared the responses of the whole population. Our post-survey had fewer respondents (147 respondents, 79% of pre-survey respondents). This difference could potentially skew our data if the post-survey population is distinct, e.g. has a higher percentage of people with baseline commitment to equity and belonging social action, leading them to take the post-survey in higher numbers. The same proportion of respondents identified as URM in both pre and post-surveys (36.3%), so differences in the overall proportion of URM respondents did not contribute to the observed differences in belonging, comfort sharing, and planned involvement in future equity efforts after the conference.

Understanding how the effects of the conference and sense of belonging may differ for individuals with specific under-represented identities or intersections of identities (e.g. Black students, Latinas), was not possible because we only collected information on whether respondents identified as an individual of an under-represented group. We chose to use the URM umbrella because requesting more specific identity information would have made responses identifiable, and may have made respondents feel less comfortable answering the survey. This approach has downsides, however, since people with specific identities have distinct experiences that may not be reflected in our data, and single-axis frameworks (e.g. looking at only race or only gender) are known to erase issues faced by people at the intersection of multiple marginalized identities (Crenshaw, 1989; Miriti, 2020; "The combahee river collective statement" (1977), n.d.). Future evaluation efforts could focus more attention on qualitative feedback and incorporate interviews to receive more individual, nuanced feedback. Cross-institutional initiatives and evaluation could also uncover experiences specific to particular identity groups with a larger sample, however, attention to differences between institutional contexts should be included in an analysis (Mertens and Hopson, 2006).

Part III

Closing Remarks

6 Discussion and Future Directions

I was drawn to study how immune activation during pregnancy impacts the brain and behavior, and sex differences in this process, because of the beautiful complexity of these systems. Brain development is a carefully orchestrated biological process that results in a range of outcomes based on genetic, environmental, hormonal, and epigenetic variables. How an organism behaves is tied to the way their brain was initially formed and is further modulated by their experience throughout life, as well as developmental processes like puberty and aging. The immune system is another complex set of organs, cell types, and processes with the amazing potential to protect us from pathogens, as well as learn and remember prior encounters. Far from being active only in response to a pathogenic threat, we've come to understand that immune cells are critical for homeostasis in the body, helping developmental processes proceed successfully and keeping the body healthily humming along. Sexual differentiation begins early in development and affects all parts of the body, including the brain. Like brain development, the process of sexual differentiation leads to a wide range of outcomes (not just two), and impacts whether an organism develops particular organs, what hormones they produce, and whether they are more likely to develop certain conditions and illnesses. Pregnancy is yet another wild ride of biological complexity, with the development of a new organ (the placenta) as well as a new organism in coordination with the pregnant organism. Studying the intersection and cross-talk between these active biological systems is an immense challenge, yet working within this complexity provides many opportunities for novel insights into the biology of one system by seeing how it is affected by another.

I also chose to work on sex differences in the immune response and neurodevelopment because of the societal implications of the work. By studying sex differences I could address years of neglect on the part of scientists only studying males. I hoped to make a positive impact on people through basic research investigating the mechanisms of neuropsychiatric conditions. These goals initially spurred me to pursue my project, and the further I got into my research, the more I began to think about other societal questions and implications.

In this final chapter, I will discuss some of the social questions that arose for me in the process of doing my dissertation work, as well as future directions for study that incorporate these perspectives.

6.1 Sex Differences in the Brain and Behavior

Sex as a biological variable has been under-studied and this neglect has led to failures in treating females, intersex people, and trans people. This deficit in research needs to be remedied (Na-

tional Institutes of Health Office of Research on Women's Health, n.d.). However, studying sex differences, particularly in the brain and behavior, also has the potential to exacerbate negative perceptions and stereotypes (Fine and Elgar, 2017). Researchers have historically applied their social understanding of gender roles when interpreting the results of studies of sex differences (Bluhm, 2013; Maney, 2016). The presence of sex difference can be used to justify differential treatment based on sex and/or gender, often via exaggerated assumptions about the nature of sex differences. Work by Joel and colleagues has shown that brain traits with sex differences often show significant overlap among populations of difference sexes, and within a given individual, characteristics that display sex differences can vary in the degree of "female-ness" (Joel, 2011; Joel et al., 2015). Therefore, when studying the brain and behavior, it is important to balance the need to demonstrate true sex differences in order to better serve people of all sexes, with the potential pitfalls of searching for sex differences to justify assumptions or over-interpreting sex differences as a dichotomy.

This picture becomes even more complicated in the context of studying biology relevant to autism spectrum disorders (ASD). ASD has historically been viewed as a male disorder, and females have often been denied participation in research as well as having lower rates of diagnosis. In this context, sex differences were assumed to exist to such an extent that they weren't even studied; non-males were simply excluded. Therefore, studying non-males, whether in human or animal studies, will help us understand how their experience or underlying biological mechanisms may be the same or different from males. To avoid the pitfalls discussed above, brain and behavior research relevant for ASD should take into account the distributions and variability in sex differences within and between individuals, rather than simply noting that sex differences exist (Maney, 2016).

In extending my behavior research in the maternal immune activation (MIA) model for ASD, I would like to use unbiased behavior screening to get a more holistic picture of the behavioral phenotype in female mice. Many of the behavior tests used in the MIA literature have been primarily tested in male animals, potentially developed with the assumption that males would have more pronounced phenotypes given the sex-bias in ASD. Long-term naturalistic behavioral video monitoring and computational analysis could shed light on whether females demonstrate behaviors in response to MIA that aren't captured by typical tests, and that may be absent or less pronounced in males (Chaumont et al., 2019; Barroca et al., 2022; Puścian and Knapska, 2022). Theories about the female protective effect in ASD suggest that higher doses of maternal immune activation stimulating compound or additional genetic or environmental "hits" may be required to detect female behavioral responses.

In addition to increased inclusion of females in ASD-related research, study of individuals with intersex taits could help broaden our understanding of ASD mechanisms and provide insight into aspects of autism specific to different sex characteristics. Research in humans has uncovered increased incidence of autism traits and/or ASD diagnoses in people with intersex characteristics, such as sex chromosome aneuploidies and polycystic ovary syndrome, as well as transgender and non-binary people assigned female at birth (Cordeiro et al., 2012; Tartaglia et al., 2017; Cherskov et al., 2018; Rijn, 2019; Kung, 2020; Warrier et al., 2020). Using animal models that help distinguish biological effects of sex chromosomes and gonadal hormones, such as the four-core-genotypes

model, could help us understand how variation in sex characteristics influences ASD etiology and phenotypes (De Vries et al., 2002). In the four-core-genotypes model, the *Sry* gene, which is required for testes formation, is moved from the Y chromosome to an autosome. This genetic change enables the creation of both XX and XY mice with or without testes, and thereby the parsing of chromosomal vs. gonadal effects. In fact, this model has been used in combination with a model for ASD that exposes fetuses to reactive maternal antibodies, finding that male bias in this model was predominantly a sex chromosome effect (Gata-Garcia et al., 2021). In future work, it would be informative to apply the fore-core-genotypes approach to the MIA model to pinpoint the role of different sex-specific mechanisms in the observed behavioral phenotypes, and clarify the role of sex chromosome gene dosage and/or sex hormone effects.

6.2 Maternal-Fetal Mechanisms Related to Autism Spectrum Disorder

Studying the effects of maternal immune activation (MIA) during pregnancy on outcomes for offspring brain and behavior has import social implications for the rights and lives of autistic people. The MIA model was developed as a way to understand the contribution of environmental and immune factors in the genesis of complex neurodevelopmental disorders like schizophrenia and autism spectrum disorder (ASD). Similar to most biomedical research with relevance to autism, research using the MIA model often represents a "medicalized" or "pathology" approach to autism (Walker and Raymaker, 2021). In this medical paradigm, autism is viewed as a disease to be treated or prevented, with deficits that represent a deviation from normal, a view which has been challenged by the neurodiversity movement (Pellicano and Houting, 2021; Walker and Raymaker, 2021). This movement promotes the idea that there is no neurological "normal," and that autism is one form of diversity in the broad range of human brains/bodies. The movement has focused attention on the societal oppression of autistic people rather than trying to change autistic individuals. Some argue that the medical and neurodiversity paradigms are inherently opposed, while others have argued for integrating medical interventions alongside the neurodiversity paradigm in order to provide needed supports for autistic people (Doyle, 2022).

Though I have attempted to remove deficit-based language that stigmatizes autistic people from descriptions of my research, the research questions are still rooted in a medical model. I don't believe autism needs to be cured, but by studying the association between maternal immune activation and autism-like phenotypes, I may inadvertently contribute to the narrative that autism research should primarily focus on causes (with the subtext of prevention) rather than the lived experiences of autistic people. In the future, I would like to focus my work on research priorities as articulated by the autism community, which include interventions to promote well-being, physical and mental health, and research into co-occuring conditions (Frazier et al., 2018; Roche et al., 2020). One promising area of study is the gut-brain axis in autism. Autistic people have higher incidence of gastrointestinal (GI) symptoms, and better understanding the nature and mechanism of these GI challenges could lead to treatments and improved quality of life for autistic people suffering with GI issues (Srikantha and Mohajeri, 2019; Settanni et al., 2021).

Ideally, autism research would include autistic people in the design, experimentation, analysis, and interpretation of results, and research output would be beneficial to the autistic community (Robertson, 2009; Gillespie-Lynch et al., 2017). An example is the community-based participatory research organized by the Academic Autism Spectrum Partnership in Research and Education (Academic Autism Spectrum Partnership in Research and Education, n.d.). Such participatory research may be more common among social scientists or at medical schools, but I propose basic biological research groups and departments should also prioritize interaction and co-leadership with communities impacted by our research. Making this happen will require personal as well as institutional buy-in, since research agendas are often set by a combination of principle investigator interests and knowledge, previous data availability, student interests and knowledge, funding priorities, and available collaborators and resources. I was able to learn more about the life experiences and research priorities of autistic people during my PhD through reading on my own and taking a student-run class on autism spectrum disorder led by autistic neuroscientist Hari Srinivasan. Still, these experiences did not meaningfully shift the research direction of my graduate work which was already underway and is partially defined by the lab environment and available resources.

Shifting scientific research to be more responsive and welcoming to the broader community is difficult, since the culture of science is often perceived as individualistic and curiosity-based at the expense of value-driven research and communal goals. However, in reflecting on my scientific training, I would have benefited from increased opportunities to apply my values to my research, collaborate with community members, and take interdisciplinary coursework that addresses axes of disability, sex and gender, and applies these social constructs to the process of science. Basic science doesn't always leave room for questions like: Why are we studying this? What biological and social frameworks are we using to look at this question? Who might be positively or negatively impacted by this work and how? I would love to see future graduate students in the sciences grapple with these questions as a standard part of their graduate training, and be supported in finding answers that they can incorporate back into their research.

6.3 Evidence-Based Pedagogy and Equity Initiatives in STEM

Through my involvement in initiatives to improve the teaching and learning environment in the MCB Department at UC Berkeley, as well as to promote equity and belonging for underrepresented scientists on campus, I've worked with an incredibly talented and caring group of people. There are many scientists with interests and commitment that extend beyond their bench to their community. Though the legacy of academic and scientific institutions is to neglect teaching and equity in favor of scientific research, these priorities are shifting with sustained effort by advocates.

In many cases we already know what needs to be done - education research has identified the importance of active learning, frequent low-stakes assessments, and authentic research experiences

in promoting equity in STEM education. Social science research has shown the importance of historically black colleges and universities and other minority-serving institutions, community-oriented research and academic support programs, policies that promote fair practices and a positive work climate, financial support, social support, quality mentorship, accessibility, and cultivation of scientific identity in making STEM a viable and attractive option for scientists from under-represented minorities.

There are hurdles to implementing these interventions, including money, time, and accountability. During my PhD I've seen some improvement in service labor being compensated, though not proportional to the expertise and effort expended, especially by women of color. Institutions that claim to value diversity and equity need to compensate those doing the work for those goals, and also need to provide adequate base salary and benefits so scientists with less access to wealth can afford to do research. Until incentive structures in academia are reorganized to better reward excellence in teaching and successful efforts to improve equity, there will be a "minority tax" on the people putting effort into these domains. Continuous program evaluation and data transparency can provide accountability for organizations seeking to improve equity.

Learning about teaching best practices and organizing for social justice have helped me grow into a well-rounded scientist. These skills are critical for STEM careers, and I propose that graduate education in the sciences could benefit from more official recognition of this work and incorporation into curricula and training. For example, students could choose to supplement their lab training with an intensive in pedagogy or pursue a leadership/organizational change track. Institutionalizing this work could spread the load to reduce the burden on people from under-represented minorities, as well as raise the profile of teaching and equity work as legitimate and necessary components of being a professional scientist.

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A Code Availability

The code used for analysis and plotting of results for this dissertation is available at https://github.com/madelinearnold/thesis.git. Data analysis and plotting was primarily performed in Quarto .qmd notebooks, creating results directly from code and integrating with text to produce the dissertation chapters. In order to simplify notebook code and reuse functions, some functions were saved in .R files and sourced. Notebooks were compiled into a PDF of the dissertation using the Quarto book format.